EXHIBIT AS

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 1 766 010 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
16.02.2011 Bulletin 2011/07

(21) Application number: **05754344.9**

(22) Date of filing: **28.06.2005**

(51) Int Cl.:
C12N 15/113 (2010.01) ***A61K 48/00*** (2006.01)
A61K 31/7105 (2006.01) ***A61K 31/711*** (2006.01)
C07H 21/00 (2006.01) *A61P 21/00* (2006.01)

(86) International application number:
PCT/AU2005/000943

(87) International publication number:
WO 2006/000057 (05.01.2006 Gazette 2006/01)

(54) **ANTISENSE OLIGONUCLEOTIDES FOR INDUCING EXON SKIPPING AND METHODS OF USE THEREOF**

ANTISENSE-OLIGONUKLEOTIDE ZUR INDUKTION VON EXON-SKIPPING SOWIE VERFAHREN ZUR VERWENDUNG DAVON

OLIGONUCLÉOTIDES ANTISENS PERMETTANT D'INDUIRE UN SAUT D'EXON ET LEURS PROCÉDÉS D'UTILISATION

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR
Designated Extension States:
HR LV

(30) Priority: **28.06.2004 AU 2004903474**

(43) Date of publication of application:
28.03.2007 Bulletin 2007/13

(60) Divisional application:
10004274.6 / 2 206 781

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(56) References cited:
WO-A1-02/24906 **WO-A1-2004/083432**
WO-A2-2004/083446 **AU-A1- 2003 284 638**
AU-B2- 780 517 **CA-A1- 2 507 125**

- **MATSUO M: "DUCHENNE AND BECKER MUSCULAR DYSTROPHY: FROM GENE DIAGNOSIS TO MOLECULAR THERAPY" IUBMB LIFE, vol. 53, no. 3, 1 March 2002 (2002-03-01), pages 147-152, XP009021242 ISSN: 1521-6543**
- **ERRINGTON STEPHEN J ET AL: "Target selection for antisense oligonucleotide induced exon skipping in the dystrophin gene." THE JOURNAL OF GENE MEDICINE, vol. 5, no. 6, June 2003 (2003-06), pages 518-527, XP002559309 ISSN: 1099-498X**
- **AARTSMA-RUS A ET AL: 'Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense' AMERICAN JOURNAL OF HUMAN GENETICS vol. 74, no. 1, 2004, pages 83 - 92, XP008084158**
- **DE ANGELIS F G ET AL: 'Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES vol. 99, no. 14, 2002, pages 9456 - 9461, XP008115971**

EP 1 766 010 B1

- **AARTSMA-RUS A ET AL: 'Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy.' NEUROMUSCULAR DISORDERS vol. 12, 2002, pages 71 - 77, XP008116183**
- **AARTSMA-RUS A ET AL: 'Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients.' HUMAN MOLECULAR GENETICS vol. 12, no. 8, 2003, pages 907 - 914, XP008084159**

Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

Description

Field of the Invention

[0001] The present invention relates to novel antisense compounds and compositions suitable for facilitating exon skipping. It also provides methods for inducing exon skipping using the novel antisense compounds as well as therapeutic compositions adapted for use in the methods of the invention..

Background Art

[0002] Significant effort is currently being expended researching methods for suppressing or compensating for disease-causing mutations in genes. Antisense technologies are being developed using a range of chemistries to affect gene expression at a variety of different levels (transcription, splicing, stability, translation). Much of that research has focused on the use of antisense compounds to correct or compensate for abnormal or disease-associated genes in a myriad of different conditions.

[0003] Antisense molecules are able to inhibit gene expression with exquisite specificity and because of this many research efforts concerning oligonucleotides as modulators of gene expression have focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The antisense oligonucleotides are directed either against RNA (sense strand) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect in specific gene down-regulation, the oligonucleotides must either promote the decay of the targeted mRNA or block translation of that mRNA, thereby effectively preventing *de novo* synthesis of the undesirable target protein.

[0004] Such techniques are not useful where the object is to up-regulate production of the native protein or compensate for mutations which induce premature termination of translation such as nonsense or frame-shifting mutations.

[0005] Furthermore, in cases where a normally functional protein is prematurely terminated because of mutations therein, a means for restoring some functional protein production through antisense technology has been shown to be possible through intervention during the splicing processes (Sierakowska H, et al., (1996) Proc Natl Acad Sci USA 93,12840-12844; Wilton SD, et al., (1999) Neuromusc Disorders 9, 330-338; van Deutekom JC et al., (2001) Human Mol Genet 10, 1547-1554). In these cases, the defective gene transcript should not be subjected to targeted degradation so the antisense oligonucleotide chemistry should not promote target mRNA decay.

[0006] In a variety of genetic diseases, the effects of mutations on the eventual expression of a gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules. It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanisms invoked have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts.

[0007] In nature, the extent of genetic deletion or exon skipping in the splicing process is not fully understood, although many instances have been documented to occur, generally at very low levels (Sherrat TG, et al., (1993) Am J Hum Genet 53, 1007-1015). However, it is recognised that if exons associated with disease-causing mutations can be specifically deleted from some genes, a shortened protein product can sometimes be produced that has similar biological properties of the native protein or has sufficient biological activity to ameliorate the disease caused by mutations associated with the target exon (Lu QL, et al., (2003) Nature Medicine 9, 1009-1014; Aartsma-Rus A et al., (2004) Am J Hum Genet 74: 83-92).

[0008] This process of targeted exon skipping is likely to be particularly useful in long genes where there are many exons and introns, where there is redundancy in the genetic constitution of the exons or where a protein is able to function without one or more particular exons (e.g. with the dystrophin gene, which consists of 79 exons; or possibly some collagen genes which encode for repeated blocks of sequence or the huge nebulin or titin genes which are comprised of -80 and over 370 exons, respectively).

[0009] Efforts to redirect gene processing for the treatment of genetic diseases associated with truncations caused by mutations in various genes have focused on the use of antisense oligonucleotides that either: (1) fully or partially overlap with the elements involved in the splicing process; or (2) bind to the pre-mRNA at a position sufficiently close to the element to disrupt the binding and function of the splicing factors that would normally mediate a particular splicing reaction which occurs at that element (e.g., binds to the pre-mRNA at a position within 3, 6, or 9 nucleotides of the

element to be blocked).

[0010] For example, modulation of mutant dystrophin pre-mRNA. splicing with antisense oligoribonucleotides has been reported both *in vitro* and *in vivo.* In one type of dystrophin mutation reported in Japan, a 52-base pair deletion mutation causes exon 19 to be removed with the flanking introns during the splicing process (Matsuo et al., (1991) J Clin Invest. 87:2127-2131). An *in vitro* minigene splicing system has been used to show that a 31-mer 2'-O-methyl oligoribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type *pre-mRNA* (Takeshima et al. (1995), J. Clin. Invest., 95, 515-520). The same oligonucleotide was used to induce exon skipping from the native dystrophin gene transcript in human cultured lymphoblastoid cells.

[0011] Dunckley et al., (1997) Nucleosides & Nucleotides, 16, 1665-1668 described *in vitro* constructs for analysis of splicing around exon 23 of mutated dystrophin in the *mdx* mouse mutant, a model for muscular dystrophy. Plans to analyse these constructs *in vitro* using 2' modified oligonucleotides targeted to splice sites within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

[0012] 2'-O-methyl oligoribonucleotides were subsequently reported to correct dystrophin deficiency in myoblasts from the *mdx* mouse from this group. An antisense oligonucleotide targeted to the 3' splice site of murine dystrophin intron 22 was reported to cause skipping of the mutant exon as well as several flanking exons and created a novel in-frame dystrophin transcript with a novel internal deletion. This mutated dystrophin was expressed in 1-2% of antisense treated mdx myotubes. Use of other oligonucleotide modifications such as 2'-O-methoxyethyl phosphodiesters are described (Dunckley et al. (1998) Human Mol. Genetics, 5, 1083-90).

[0013] Thus, antisense molecules may provide a tool in the treatment of genetic disorders such as Duchenne Muscular Dystrophy (DMD). However, attempts to induce exon skipping using antisense molecules have had mixed success. Studies on dystrophin exon 19, where successful skipping of that exon from the dystrophin pre-mRNA was achieved using a variety of antisense molecules directed at the flanking splice sites or motifs within the exon involved in exon definition as described by Errington et al. (2003) J Gen Med 5, 518-527".

[0014] In contrast to the apparent ease of exon 19 skipping, the first report of exon 23 skipping in the *mdx* mouse by Dunckley *et al.,* (1998) is now considered to be reporting only a naturally occurring revertant transcript or artefact rather than any true antisense activity. In addition to not consistently generating transcripts missing exon 23, Dunckley *et al.,* (1998) did not show any time course of induced exon skipping, or even titration of antisense oligonucleotides, to demonstrate dose dependent effects where the levels of exon skipping corresponded with increasing or decreasing amounts of antisense oligonucleotide. Furthermore, this work could not be replicated by other researchers.

[0015] The first example of specific and reproducible exon skipping in the *mdx* mouse model was reported by Wilton et al., (1999) Neuromuscular Disorders 9, 330-338. By directing an antisense molecule to the donor splice site, consistent and efficient exon 23 skipping was induced in the dystrophin mRNA within 6 hours of treatment of the cultured cells. Wilton et al., (1999), also describe targeting the acceptor region of the mouse dystrophin pre-mRNA with longer antisense oligonucleotides and being unable to repeat the published results of Dunckley et al., (1998). No exon skipping, either 23 alone or multiple removal of several flanking exons, could be reproducibly detected using a selection of antisense oligonucleotides directed at the acceptor splice site of intron 22.

[0016] While the first antisense oligonucleotide directed at the intron 23 donor splice site induced consistent exon skipping in primary cultured myoblasts, this compound was found to be much less efficient in immortalized cell cultures expressing higher levels of dystrophin. However, with refined targeting and antisense oligonucleotide design, the efficiency of specific exon removal was increased by almost an order of magnitude (see Mann CJ et al., (2002) J Gen Med 4, 644654).

[0017] Other disclosures relating to DMD therapy include CA 2507125 and Matsuo M: "Duchenne And Becker Muscular Dystrophy: From Gene Diagnosis To Molecular Therapy" IUBMB LIFE, vol.53, no. 3, 1 March 2002 (2002-03-01), pages 147-152, as well as WO 2004/083446, published on 30 September 2004.

[0018] Thus, there remains a need to provide antisense oligonucleotides capable of binding to and modifying the splicing of a target nucleotide sequence. Simply directing the antisense oligonucleotides to motifs presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.

Summary of the Invention

[0019] The present invention provides an antisense oligonucleotide that binds to the human dystrophin gene to induce exon skipping in the dystrophin gene, consisting of the sequence of SEQ ID NO: 181, optionally wherein the uracil bases (U) are thymine bases (T).

[0020] The invention further provides a composition comprising an antisense molecule according to the invention one or more pharmaceutically acceptable carriers and/or diluents.

[0021] The invention further provides an antisense molecule or composition according to the invention for use in a method of treatment of muscular dystrophy in a patient.

[0022] The invention is further defined in the accompanying claims.

[0023] The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to target regions of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are the donor and acceptor splice sites although there are less defined or conserved motifs including exonic splicing enhancers, silencing elements and branch points.

[0024] The acceptor and donor splice sites have consensus sequences of about 16 and 8 bases respectively (see Figure 1 for schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process).

[0025] The invention also addresses the use of purified and isolated antisense oligonucleotides of the invention, for the manufacture of a medicament for treatment of a genetic disease.

[0026] The invention may be used for treating a condition characterised by Duchenne muscular dystrophy, by administering to a patient in need of treatment an effective amount of an appropriately designed antisense oligonucleotide of the invention, relevant to the particular genetic lesion in that patient.

[0027] Further, the invention may be used for prophylactically treating a patient to prevent or at least minimise Duchene muscular dystrophy, comprising the step of: administering to the patient an effective amount of an antisense oligonucleotide or a pharmaceutical composition comprising one or more of these biological molecules.

[0028] Also described herein are kits for treating a genetic disease, which kits comprise at least a antisense oligonucleotide of the present invention, packaged in a suitable container and instructions for its use.

[0029] Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following figures.

Brief Description of the Drawings

[0030]

Figure 1 Schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process.

Figure 2. Diagrammatic representation of the concept of antisense oligonucleotide induced exon skipping to bypass disease-causing mutations (not drawn to scale). The hatched box represents an exon carrying a mutation that prevents the translation of the rest of the mRNA into a protein. The solid black bar represents an antisense oligonucleotide that prevents inclusion of that exon in the mature mRNA.

[0031] The 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to study induced exon skipping during the processing of the dystrophin pre-mRNA are described in Table 1 of the examples. Since these 2'-O-methyl antisense oligonucleotides are more RNA-like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as "T".

Detailed Description of the Invention

General

[0032] Those skilled In the art will appreciate that the Invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0033] The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

[0034] Sequence identity numbers (SEQ ID NO:) containing nucleotide and amino acid sequence information included in this specification are collected at the end of the description and have been prepared using the programme Patentin Version 3.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc.). The length, type of sequence and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided In numeric indicator field <400> followed by the sequence identifier (e.g. <400>1, <400>2, etc.).

[0035] An antisense molecules nomenclature system was proposed and published to distinguish between the different

antisense molecules (see Mann et al., (2002) J Gen Med 4, 644-654). This nomenclature became especially relevant when testing several slightly different antisense molecules, all directed at the same target region, as shown below:

H # A/D (x : y).

[0036] The first letter designates the species (e.g. H: human, M: murine, C: canine)
"#" designates target dystrophin exon number.

[0037] "A/D" indicates acceptor or donor splice site at the beginning and end of the exon, respectively.

[0038] (x y) represents the annealing coordinates where "-" or "+" indicate intronic or exonic sequences respectively. As an example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest splice site would be the acceptor so these coordinates would be preceded with an "A". Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by A(+65+85), that is the site between the 65th and 85th nucleotide from the start of that exon.

[0039] No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working In the field to which this invention relates.

[0040] As used necessarily herein the term "derived" and "derived from" shall be taken to Indicate that a specific integer may be obtained from a particular source *albeit* not directly from that source.

[0041] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0042] Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Description of the Preferred Embodiment

[0043] When antisense molecule(s) are targeted to nucleotide sequences involved in splicing in exons within pre-mRNA sequences, normal splicing of the exon may be inhibited causing the splicing machinery to by-pass the entire mutated exon from the mature mRNA. The concept of antisense oligonucleotide induced exon skipping Is shown in Figure 2. In many genes, deletion of an entire exon would lead to the production of a non-functional protein through the loss of important functional domains or the disruption of the reading frame. In some proteins, however, it is possible to shorten the protein by deleting one or more exons, without disrupting the reading frame, from within the protein without seriously altering the biological activity of the protein. Typically, such proteins have a structural role and or possess functional domains at their ends. The present invention describes antisense molecules capable of binding to specified dystrophin pre-mRNA targets and re-directing processing of that gene.

Antisense Molecules

[0044] According to a first aspect of the invention, there is provided antisense molecules as defined in the claims capable of binding to a selected target to Induce exon skipping. Designing antisense molecules to completely mask consensus splice sites may not necessarily generate any skipping of the targeted exon. Furthermore, the inventors have discovered that size or length of the antisense oligonucleotide itself is not always a primary factor when designing antisense molecules. With some targets such as exon 19, antisense oligonucleotides as short as 12 bases were able to induce exon skipping, albeit not as efficiently as longer (20-31 bases) oligonucleotides. In some other targets, such as murine dystrophin exon 23, antisense oligonucleotides only 17 residues long were able to induce more efficient skipping than another overlapping compound of 25 nucleotides.

[0045] The inventors have also discovered that there does not appear to be any standard motif that can be blocked or masked by antisense molecules to redirect splicing. In some exons, such as mouse dystrophin exon 23, the donor splice site was the most amenable to target to re-direct skipping of that exon. It should be noted that designing and testing a series of exon 23 specific antisense molecules to anneal to overlapping regions of the donor splice site showed considerable variation in the efficacy of induced exon skipping. As reported In Mann et al., (2002) there was a significant variation in the efficiency of bypassing the nonsense mutation depending upon antisense oligonucleotide annealing ("improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy". J Gen Med 4: 644-654). Targeting the acceptor site of exon 23 or several internal domains was not found to Induce any consistent exon 23 skipping.

[0046] In other exons targeted for removal, masking the donor splice site did not induce any exon skipping. However, by directing antisense molecules to the acceptor splice site (human exon 8) strong and sustained exon skipping was induced. It should be noted that removal of human exon 8 was tightly linked with the co-removal of exon 9. There is no strong sequence homology between the exon 8 antisense oligonucleotides and corresponding regions of exon 9 so it does not appear to be a matter of cross reaction. Rather the splicing of these two exons is inextricably linked. This is not an isolated instance as the same effect is observed in canine cells where targeting exon 8 for removal also resulted in the skipping of exon 9. Targeting exon 23 for removal in the mouse dystrophin pre-mRNA also results in the frequent removal of exon 22 as well. This effect occurs in a dose dependent manner and also indicates close coordinated processing of 2 adjacent exons.

[0047] In other targeted exons, antisense molecules directed at the donor or acceptor splice sites did not induce exon skipping while annealing antisense molecules to intra-exonic regions (i.e. exon splicing enhancers within human dystrophin exon 6) was most efficient at inducing exon skipping. Some exons, both mouse and human exon 19 for example, are readily skipped by targeting antisense molecules to a variety of motifs. That is, targeted exon skipping Is induced after using antisense oligonucleotides to mask donor and acceptor splice sites or exon splicing enhancers.

[0048] To identify and select antisense oligonucleotides suitable for use in the modulation of exon skipping, a nucleic acid sequence whose function is to be modulated must first be identified. This may be, for example, a gene (or mRNA transcribed form the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites, or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0049] Preferably, the present invention aims to provide antisense molecules capable of binding to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping. Duchenne muscular dystrophy arises from mutations that preclude the synthesis of a functional dystrophin gene product. These Duchenne muscular dystrophy gene defects are typically nonsense mutations or genomic rearrangements such as deletions, duplications or micro-deletions or insertions that disrupt the reading frame. As the human dystrophin gene is a large and complex gene with the 79 exons being spliced together to generate a mature mRNA with an open reading frame of approximately 11,000 bases, there are many positions where these mutations can occur. Consequently, a comprehensive antisense oligonucleotide based therapy to address many of the different disease-causing mutations in the dystrophin gene will require that many exons can be targeted for removal during the splicing process.

[0050] Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0051] The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridisable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable. An antisense molecule is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

[0052] While the above method may be used to select antisense molecules capable of deleting any exon from within a protein that is capable of being shortened without affecting its biological function, the exon deletion should not lead to a reading frame shift in the shortened transcribed mRNA. Thus, if in a linear sequence of three exons the end of the first exon encodes two of three nucleotides in a codon and the next exon is deleted then the third exon in the linear sequence must start with a single nucleotide that is capable of completing the nucleotide triplet for a codon. If the third exon does not commence with a single nucleotide there will be a reading frame shift that would lead to the generation of truncated or a non-functional protein.

[0053] It will be appreciated that the codon arrangements at the end of exons in structural proteins may not always break at the end of a codon, consequently there may be a need to delete more than one exon from the pre-mRNA to ensure in-frame reading of the mRNA. In such circumstances, a plurality of antisense oligonucleotides may need to be selected by the method of the invention wherein each is directed to a different region responsible for inducing splicing in the exons that are to be deleted.

[0054] The length of an antisense molecule may vary so long as it is capable of binding selectively to the intended location within the pre-mRNA molecule. The length of such sequences can be determined in accordance with selection procedures described herein. Generally, the antisense molecule will be from about 10 nucleotides in length up to about

50 nucleotides in length. It will be appreciated however that any length of nucleotides within this range may be used in the method. Preferably, the length of the antisense molecule is between 17 to 30 nucleotides in length.

[0055] In order to determine which exons can be connected in a dystrophin gene, reference should be made to an exon boundary map. Connection of one exon with another is based on the exons possessing the same number at the 3' border as is present at the 5' border of the exon to which it is being connected. Therefore, if exon 7 were deleted, exon 6 must connect to either exons 12 or 18 to maintain the reading frame. Thus, antisense oligonucleotides would need to be selected which redirected splicing for exons 7 to 11 in the first instance or exons 7 to 17 in the second instance. Another and somewhat simpler approach to restore the reading frame around an exon 7 deletion would be to remove the two flanking exons. Induction of exons 6 and 8 skipping should result in an in-frame transcript with the splicing of exons 5 to 9. In practise however, targeting exon 8 for removal from the pre-mRNA results in the co-removal of exon 9 so the resultant transcript would have exon 5 joined to exon 10. The inclusion or exclusion of exon 9 does not after the reading frame. Once the antisense molecules to be tested have been identified, they are prepared according to standard techniques known in the art. The most common method for producing antisense molecules is the methylation of the 2' hydroxyribose position and the incorporation of a phosphorothioate backbone produces molecules that superficially resemble RNA but that are much more resistant to nuclease degradation.

[0056] To avoid degradation of pre-mRNA during duplex formation with the antisense molecules, the antisense molecules used in the method may be adapted to minimise or prevent cleavage by endogenous RNase H. This property is highly preferred as the treatment of the RNA with the unmethylated oligonucleotides either intracellularly or in crude extracts that contain RNase H leads to degradation of the pre-mRNA: antisense oligonucleotide duplexes. Any form of modified antisense molecules that is capable of by-passing or not inducing such degradation may be used in the present method. An example of antisense molecules which when duplexed with RNA are not cleaved by cellular RNase H is 2'-O-methyl derivatives. 2'-O-methyl-oligoribonucleotides are very stable in a cellular environment and in animal tissues, and their duplexes with RNA have higher Tm values than their ribo- or deoxyribo- counterparts.

[0057] Antisense molecules that do not activate RNase H can be made in accordance with known techniques (see, e.g., U.S. Pat. 5,149,797). Such antisense molecules, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the portions of the oligonucleotide involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense molecules that do not activate RNase H are available. For example, such antisense molecules may be oligonucleotides wherein at least one, or all, of the inter-nucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another nonlimiting example, such antisense molecules are molecules wherein at least one, or all, of the nucleotides contain a 2' lower alkyl moiety (e.g., C_1-C_4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, every other one of the nucleotides may be modified as described.

[0058] While antisense oligonucleotides are a preferred form of the antisense molecules, the present invention comprehends other oligomeric antisense molecules, including but not limited to oligonucleotide mimetics such as are described below.

[0059] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

[0060] In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleo-bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

[0061] Modified oligonucleotides may also contain one or more substituted sugar moieties. Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. Certain nucleo-bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0062] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

[0063] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense molecules, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the increased resistance to nuclease degradation, increased cellular uptake, and an additional region for increased binding affinity for the target nucleic acid.

Methods of Manufacturing Antisense Molecules

[0064] The antisense molecules used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). One method for synthesising oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

[0065] Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. In one such automated embodiment, diethyl-phosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al., (1981) Tetrahedron Letters, 22:1859-1862.

[0066] The antisense molecules of the invention are synthesised *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules. The molecules of the invention may also be mixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

Therapeutic Agents

[0067] The present invention also can be used as a prophylactic or therapeutic, which may be utilised for the purpose of treatment of a genetic disease.

[0068] Accordingly, in one embodiment the present invention provides antisense molecules that bind to a selected target In the dystrophin pre-mRNA to Induce efficient and consistent exon skipping described herein in a therapeutically effective amount admixed with a pharmaceutically acceptable carrier, diluent, or excipient.

[0069] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to a patient. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for Injectable solutions. Suitable pharmaceutical carriers are described In Martin, Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

[0070] In a more specific form of the invention there are provided pharmaceutical compositions comprising therapeutically effective amounts of an antisense molecule together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength and additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.*, Thimersol, benzyl alcohol) and bulking substances (*e.g.*, lactose, mannitol). The material may be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.,* Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilised form.

[0071] It will be appreciated that pharmaceutical compositions provided according to the present invention may be administered by any means known in the art. Preferably, the pharmaceutical compositions for administration are administered by injection, orally, or by the pulmonary, or nasal route. The antisense molecules are more preferably delivered by intravenous, intra-arterial, intraperitoneal, intramuscular, or subcutaneous routes of administration.

Antisense molecule based therapy

[0072] Also addressed by the present invention is the use of antisense molecules of the present invention, for manufacture of a medicament for modulation of a genetic disease.

[0073] The delivery of a therapeutically useful amount of antisense molecules may be achieved by methods previously published. For example, intracellular delivery of the antisense molecule may be via a composition comprising an admixture of the antisense molecule and an effective amount of a block copolymer. An example of this method is described in US patent application US 20040248833.

[0074] Other methods of delivery of antisense molecules to the nucleus are described in Mann CJ et al., (2001) ["Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse". Proc., Natl. Acad. Science, 98 (1) 42-47] and in Gebski et al., (2003). Human Molecular Genetics, 12(15): 1801-1811.

[0075] A method for introducing a nucleic acid molecule into a cell by way of an expression vector either as naked DNA or complexed to lipid carriers, is described in US patent US 6,806,084.

[0076] It may be desirable to deliver the antisense molecule in a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations.

[0077] Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo.* These formulations may have net cationic, anionic or neutral charge characteristics and are useful characteristics with *in vitro, in vivo* and *ex vi*vo delivery methods. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 .PHI.m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, and DNA can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., Trends Biochem. Sci., 6:77, 1981).

[0078] In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the antisense molecule of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, et al., Biotechniques, 6:682, 1988).

[0079] The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

[0080] Alternatively, the antisense construct may be combined with other pharmaceutically acceptable carriers or diluents to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

[0081] The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition.Multiple approaches for introducing functional new genetic material into cells, both in vitro and in vivo have been attempted (Friedmann (1989) Science, 244:1275-1280).

[0082] These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) supra; Rosenberg (1991) Cancer Research 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, et al. (1992) Cell, 68:143-155; Rosenfeld, et al. (1991) Science, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), supra; Brigham, et al. (1989) Am. J. Med. Sci., 298:278-281; Nabel, et al. (1990) Science, 249:1285-1288; Hazinski, et al. (1991) Am. J. Resp. Cell Molec. Biol., 4:206-209; and Wang and Huang (1987) Proc. Natl. Acad. Sci. (USA), 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J. Biol. Chem., 263:14621-14624) or the use of naked DNA, expression vectors (Nabel et al. (1990), supra); Wolff et al. (1990) Science, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) supra); Rosenfeld et al. (1991) supra; Brigham et al. (1989) supra; Nabel (1990) supra; and Hazinski et al. (1991) supra). The Brigham et al. group (Am. J. Med. Sci. (1989) 298: 278-281 and Clinical Research (1991) 39 (abstract)) have reported in vivo transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, Science (1992) 256:808-813.

[0083] The antisense molecules of the invention encompass any pharmaceutically acceptable salts, esters, or salts

of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such pro-drugs, and other bioequivalents.

[0084] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

[0085] For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, (including by nebulizer, intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

[0086] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Kits of the Invention

[0087] The invention also provides kits for treatment of a patient with a genetic disease which kit comprises at least an antisense molecule, packaged in a suitable container, together with instructions for its use.

[0088] In a preferred embodiment, the kits will contain at least one antisense molecule. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

[0089] Those of ordinary skill in the field should appreciate that applications of the above method has wide application for identifying antisense molecules suitable for use in the treatment of many other diseases.

EXAMPLES

[0090] The following Examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these Examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

[0091] Methods of molecular cloning, immunology and protein chemistry, which are not explicitly described in the following examples, are reported In the literature and are known by those skilled in the art. General texts that described conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art, included, for example: Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover ed., DNA Cloning: A Practical Approach, Volumes I and II, MRL Press, Ltd., Oxford, U.K. (1985); and Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. Current Protocols In Molecular Biology. Greene Publishing Associates/Wiley Intersciences, New York (2002).

Determining Induced Exon Skipping in Human Muscle Cells

[0092] Attempts by the inventors to develop a rational approach in antisense molecules design were not completely successful as there did not appear to be a consistent trend that could be applied to all exons. As such, the identification of the most effective and therefore most therapeutic antisense molecules compounds has been the result of empirical studies.

[0093] These empirical studies involved the use of computer programs to identify motifs potentially involved in the splicing process. Other computer programs were also used to identify regions of the pre-mRNA which may not have

had extensive secondary structure and therefore potential sites for annealing of antisense molecules. Neither of these approaches proved completely reliable in designing antisense oligonucleotides for reliable and efficient induction of exon skipping.

[0094] Annealing sites on the human dystrophin pre-mRNA were selected for examination, initially based upon known or predicted motifs or regions involved in splicing. 2OMe antisense oligonucleotides were designed to be complementary to the target sequences under investigation and were synthesised on an Expedite 8909 Nucleic Acid Synthesiser. Upon completion of synthesis, the oligonucleotides were cleaved from the support column and de-protected in ammonium hydroxide before being desalted. The quality of the oligonucleotide synthesis was monitored by the intensity of the trityl signals upon each deprotection step during the synthesis as detected in the synthesis log. The concentration of the antisense oligonucleotide was estimated by measuring the absorbance of a diluted aliquot at 260nm.

[0095] Specified amounts of the antisense molecules were then tested for their ability to induce exon skipping in an *in vitro* assay, as described below.

[0096] Briefly, normal primary myoblast cultures were prepared from human muscle biopsies obtained after informed consent. The cells were propagated and allowed to differentiate into myotubes using standard culturing techniques. The cells were then transfected with the antisense oligonucleotides by delivery of the oligonucleotides to the cells as cationic lipoplexes, mixtures of antisense molecules or cationic liposome preparations.

[0097] The cells were then allowed to grow for another 24 hours, after which total RNA was extracted and molecular analysis commenced. Reverse transcriptase amplification (RT-PCR) was undertaken to study the targeted regions of the dystrophin pre-mRNA or induced exonic re-arrangements.

[0098] For example, in the testing of an antisense molecule for inducing exon 19 skipping the RT-PCR test scanned several exons to detect Involvement of any adjacent exons. For example, when inducing skipping of exon 19, RT-PCR was carried out with primers that amplified across exons 17 and 21. Amplifications of even larger products in this area (i.e. exons 13-26) were also carried out to ensure that there was minimal amplification bias for the shorter induced skipped transcript. Shorter or exon skipped products tend to be amplified more efficiently and may bias the estimated of the normal and induced transcript.

[0099] The sizes of the amplification reaction products were estimated on an agarose gel and compared against appropriate size standards. The final confirmation of identity of these products was carried out by direct DNA sequencing to establish that the correct or expected exon junctions have been maintained.

[0100] Once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the assay as described above. Our definition of an efficient antisense molecule is one that induces strong and sustained exon skipping at transfection concentrations in the order of 300 nM or less.

Antisense Oligonucleotides Directed at Exon 51

[0101] Antisense oligonucleotides directed at exon 51 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

[0102] Antisense oligonucleotide H51A(+66+90) [SEQ ID NO:180] showed the stronger ability to Induce exon 51 skipping. Table 1 below includes antisense molecules tested at a concentration range of 25, 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 51 skipping. The strongest inducers of exon skipping were antisense oligonucleotide H51A(+61+90) [SEQ ID NO: 179] and H51A(+66+95) [SEQ ID NO: 181].

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H51A(-01+25)	ACC AGA GUA ACA GUC UGA GUA GGA GC	Faint skipping
H51D(+16-07)	CUC AUA CCU UCU GCU UGA UGA UC	Skipping at 300 nM
H51A(+111+134)	UUC UGU CCA AGC CCG GUU GAA AUC	Needs retesting
H51A(+61+90)	ACA UCA AGG AAG AUG GCA UUU CUA GUU UGG	Very strong skipping
H51A(+66+90)	ACA UCA AGG AAG AUG GCA UUU CUA G	skipping
H51A(+66+95)	CUC CAA CAU CAA GGA AGA UGG CAU UUC UAG	Very strong skipping
H51D(+08-17)	AUC AUU UUU UCU CAU ACC UUC UGC U	No skipping

(continued)

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H51A/D(+08-17) & (-15+?)	AUC AUU UUU UCU CAU ACC UUC UGC UAG GAG CUA AAA	No skipping
H51A(+175+195)	CAC CCA CCA UCA CCC UCY GUG .	No skipping
H51A(+199+220)	AUC AUC UCG UUG AUA UCC UCA A	No skipping

SEQUENCE LISTING

[0103]

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Claims

1. An antisense oligonucleotide that binds to the human dystrophin gene to induce exon skipping in the dystrophin gene, consisting of the sequence of SEQ ID NO: 181, optionally wherein the uracil bases (U) are thymine bases (T).

2. An isolated antisense oligonucleotide of up to 50 nucleotides in length, wherein the antisense oligonucleotide comprises the antisense oligonucleotide of claim 1.

3. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide comprises a modified backbone or non-natural internucleotide linkages.

4. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

5. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide does not activate RNase H.

6. The antisense oligonucleotide of claim 3, wherein the sugar moieties of the oligonucleotide backbone are replaced with non-natural moieties.

7. The antisense oligonucleotide of claim 3, wherein the modified backbone comprises morpholinos.

8. The antisense oligonucleotide of claim 1, wherein the inter-nucleotide linkages of the oligonucleotide backbone are replaced with non-natural inter-nucleotide linkages.

9. The antisense oligonucleotide of claim 8, wherein the non-natural inter-nucleotide linkages are modified phosphates.

10. The antisense oligonucleotide of claim 9, wherein the modified phosphates are selected from methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates, and phosphoroamidates.

11. The antisense oligonucleotide of claim 10, wherein the modified phosphates are selected from phosphoroamidates.

12. The antisense oligonucleotide of claim 10 wherein the modified phosphates are selected from phosphoromorpholidates.

13. The antisense oligonucleotide of claim 10, wherein the modified phosphates are selected from phosphoropiperazidates.

14. The antisense oligonucleotide of claim 1, wherein the sugar moieties of the oligonucleotide backbone are replaced with non-natural moieties and the inter-nucleotide linkages of the oligonucleotide backbone are replaced with non-natural inter-nucleotide linkages.

15. The antisense oligonucleotide of any of claims 1-14, wherein the uracil bases are thymine bases.

16. The antisense oligonucleotide of claim 15, wherein the antisense oligonucleotide is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

17. The antisense oligonucleotide of claim 16, wherein the antisense oligonucleotide is chemically linked to a polyethylene glycol chain.

18. A composition comprising an antisense oligonucleotide according to any one of the preceding claims and one or more pharmaceutically acceptable carriers and/or diluents.

19. An antisense oligonucleotide according to any one of claims I to 17 or a composition according to claim 18 for use in a method of treatment of muscular dystrophy in a patient.

Patentansprüche

1. Ein Antisense-Oligonukleotid, das an humanes Dystrophin-Gen bindet, um das Überspringen von Exons in dem Dystrophin-Gen zu induzieren, das aus der Sequenz mit SEQ ID NO: 181 besteht, wobei optional die Uracil-Basen (U) Thymin-Basen (T) sind.

2. Ein isoliertes Antisense-Oligonukleotid mit einer Länge von bis zu 50 Nukleotiden, wobei das Antisense-Oligonukleotid das Antisense-Oligonukleotid nach Anspruch 1 umfasst.

3. Das Antisense-Oligonukleotid nach Anspruch 1, wobei das Antisense-Oligonukleotid ein modifiziertes Rückgrat oder unnatürliche Inter-Nukleotid-Bindungen umfasst.

4. Das Antisense-Oligonukleotid nach Anspruch 1, wobei das Antisense-Oligonukleotid chemisch an eine oder mehrere Einheiten oder Konjugate gebunden ist, die die Aktivität, die zelluläre Verteilung oder die zelluläre Aufnahme des Antisense-Oligonukleotids verstärken.

5. Das Antisense-Oligonukleotid nach Anspruch 1, wobei das Antisense-Oligonukleotid RNase H nicht aktiviert.

6. Das Antisense-Oligonukleotid nach Anspruch 3, wobei die Zucker-Einheiten des Oligonukleotid-Rückgrats mit unnatürlichen Einheiten ersetz werden.

7. Das Antisense-Oligonukleotid nach Anspruch 3, wobei das modifizierte Rückgrat Morpholinos umfasst.

8. Das Antisense-Oligonukleotid nach Anspruch 1, wobei die Inter-Nukleotid-Bindungen des Oligonukleotid-Rückgrats mit unnatürlichen Inter-Nukleotid-Bindungen ersetzt werden.

9. Das Antisense-Oligonukleotid nach Anspruch 8, wobei die unnatürlichen Inter-Nukleotid-Bindungen modifizierte Phosphate sind.

10. Das Antisense-Oligonukleotid nach Anspruch 9, wobei die modifizierten Phosphate aus Methylphosphonaten, Methylphosporothioaten, Phosphoromorpholidaten, Phosphoropiperazidaten und Phosphoroamidaten ausgewählt werden.

11. Das Antisense-Oligonukleotid nach Anspruch 10, wobei die modifizierten Phosphate aus Phosphoroamidaten ausgewählt werden.

12. Das Antisense-Oligonukleotid nach Anspruch 10, wobei die modifizierten Phosphate aus Phosphoromorpholidaten ausgewählt werden.

13. Das Antisense-Oligonukleotid nach Anspruch 10, wobei die modifizierten Phosphate aus Phosphoropiperazidaten ausgewählt werden.

14. Das Antisense-Oligonukleotid nach Anspruch 1, wobei die Zucker-Einheiten des Oligonukleotid-Rückgrats mit unnatürlichen Einheiten ersetzt werden und die Inter-Nukleotid-Bindungen des Oligonukleotid-Rückgrats mit unnatürlichen Inter-Nukleotid-Bindungen ersetzt werden.

15. Das Antisense-Oligonukleotid nach einem der Ansprüche 1-14, wobei die Uracil-Basen Thymin-Basen sind.

16. Das Antisense-Oligonukleotid nach Anspruch 15, wobei das Antisense-Oligonukleotid chemisch an eine oder mehrere Einheiten oder Konjugate gebunden ist, die die Aktivität, die zelluläre Verteilung oder die zelluläre Aufnahme des Antisense-Oligonukleotids verstärken.

17. Das Antisense-Oligonukleotid nach Anspruch 16, wobei das Antisense-Oligonukleotid chemisch an eine Polyethylenglykol-Kette gebunden ist.

18. Eine Zusammensetzung, die ein Antisense-Oligonukleotid gemäß einem der vorangegangenen Ansprüche und einen oder mehrere pharmazeutisch geeignete Träger und/oder Verdünnungsmittel umfasst.

19. Ein Antisense-Oligonukleotid gemäß einem der Ansprüche 1 bis 17 oder eine Zusammensetzung gemäß Anspruch 18 für die Verwendung in einem Verfahren zur Behandlung von muskulärer Dystrophie bei einem Patienten.

Revendications

1. Oligonucléotide antisens qui se lie au gène de dystrophine humaine pour induire un saut d'exon dans le gène de dystrophine, consistant en la séquence de SEQ ID NO : 181, éventuellement où les bases de type uracile (U) sont des bases thymines (T).

2. Oligonucléotide antisens isolé d'une longueur de jusqu'à 50 nucléotides, où l'oligonucléotide antisens comprend l'oligonucléotide antisens selon la revendication 1.

3. Oligonucléotide antisens selon la revendication 1 où l'oligonucléotide antisens comprend un squelette modifié ou des liaisons internucléotidiques non naturelles.

4. Oligonucléotide antisens selon la revendication 1 où l'oligonucléotide antisens est lié chimiquement à un ou plusieurs groupements ou conjugués qui augmentent l'activité, la distribution cellulaire ou l'absorption cellulaire de l'oligonucléotide antisens.

5. Oligonucléotide antisens selon la revendication 1 où l'oligonucléotide antisens n'active pas la RNase H.

6. Oligonucléotide antisens selon la revendication 3 où les groupements glucidiques du squelette de l'oligonucléotide sont remplacés par des groupements non naturels.

7. Oligonucléotide antisens selon la revendication 3 où le squelette modifié comprend des morpholinos.

8. Oligonucléotide antisens selon la revendication 1 où les liaisons internucléotidiques du squelette de l'oligonucléotide sont remplacées par des liaisons internucléotidiques non naturelles.

9. Oligonucléotide antisens selon la revendication 8 où les liaisons internucléotidiques non naturelles sont des phosphates modifiés.

10. Oligonucléotide antisens selon la revendication 9 où les phosphates modifiés sont choisis parmi les méthyl phosphonates, les méthyl phosphorothioates, les phosphoromorpholidates, les phosphoropipérazidates et les phosphoramidates.

11. Oligonucléotide antisens selon la revendication 10 où les phosphates modifiés sont choisis parmi les phosphoramidates.

12. Oligonucléotide antisens selon la revendication 10 où les phosphates modifiés sont choisis parmi les phosphoromorpholidates.

13. Oligonucléotide antisens selon la revendication 10 où les phosphates modifiés sont choisis parmi les phosphoropipérazidates.

14. Oligonucléotide antisens selon la revendication 1 où les groupements glucidiques du squelette de l'oligonucléotide sont remplacés par des groupements non naturels et les liaisons internucléotidiques du squelette de l'oligonucléotide sont remplacées par des liaisons internucléotidiques non naturelles.

15. Oligonucléotide antisens selon l'une quelconque des revendications 1-14 où les bases de type uracile sont des bases thymines.

16. Oligonucléotide antisens selon la revendication 15 où l'oligonucléotide antisens est lié chimiquement à un ou plusieurs groupements ou conjugués qui augmentent l'activité, la distribution cellulaire ou l'absorption cellulaire de l'oligonucléotide antisens.

17. Oligonucléotide antisens selon la revendication 16 où l'oligonucléotide antisens est lié chimiquement à une chaîne de polyéthylèneglycol.

18. Composition comprenant un oligonucléotide antisens selon l'une quelconque des revendications précédentes et un ou plusieurs vecteurs et/ou diluants pharmaceutiquement acceptables.

19. Oligonucléotide antisens selon l'une quelconque des revendications 1 à 17 ou composition selon la revendication 18 destiné(e) à être utilisé(e) dans un procédé de traitement de la dystrophie musculaire chez un patient.

FIGURE 1.

bp Acceptor ESE Donor

ucaugcacugagugaccucuuucucgcagGCGCUAGCUGGAGCA/////CCGUGCAGACUGACGgucucau

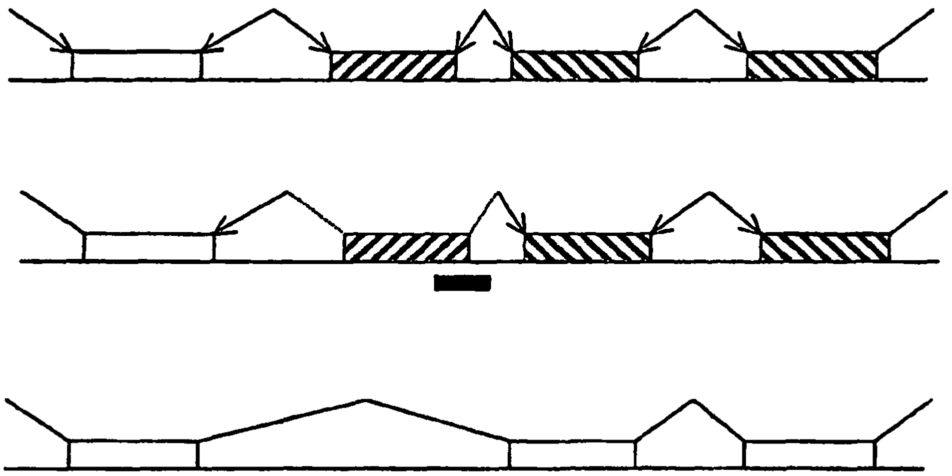

FIGURE 2

REFERENCES CITED IN THE DESCRIPTION

Patent documents cited in the description

- WO 2004083446 A **[0017]**
- US 5149797 A **[0057]**
- US 4458066 A **[0064]**
- US 20040248833 A **[0073]**
- US 6806084 B **[0075]**

Non-patent literature cited in the description

- **Sierakowska H et al.** *Proc Natl Acad Sci USA,* 1996, vol. 93, 12840-12844 **[0005]**
- **Wilton SD et al.** *Neuromusc Disorders,* 1999, vol. 9, 330-338 **[0005]**
- **van Deutekom JC et al.** *Human Mol Genet,* 2001, vol. 10, 1547-1554 **[0005]**
- **Sherrat TG et al.** *Am J Hum Genet,* 1993, vol. 53, 1007-1015 **[0007]**
- **Lu QL et al.** *Nature Medicine,* 2003, vol. 9, 1009-1014 **[0007]**
- **Aartsma-Rus A et al.** *Am J Hum Genet,* 2004, vol. 74, 83-92 **[0007]**
- **Matsuo et al.** *J Clin Invest.,* 1991, vol. 87, 2127-2131 **[0010]**
- **Takeshima et al.** *J. Clin. Invest.,* 1995, vol. 95, 515-520 **[0010]**
- **Dunckley et al.** *Nucleosides & Nucleotides,* 1997, vol. 16, 1665-1668 **[0011]**
- **Dunckley et al.** *Human Mol. Genetics,* 1998, vol. 5, 1083-90 **[0012]**
- **Errington et al.** *J Gen Med,* 2003, vol. 5, 518-527 **[0013]**
- **Wilton et al.** *Neuromuscular Disorders,* 1999, vol. 9, 330-338 **[0015]**
- **Mann CJ et al.** *J Gen Med,* 2002, vol. 4, 644654 **[0016]**
- **Matsuo M.** Duchenne And Becker Muscular Dystrophy: From Gene Diagnosis To Molecular Therapy. *IUBMB LIFE,* 01 March 2002, vol. 53 (3), 147-152 **[0017]**
- **Mann et al.** *J Gen Med,* 2002, vol. 4, 644-654 **[0035]**
- improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. *J Gen Med,* vol. 4, 644-654 **[0045]**
- **Beaucage et al.** *Tetrahedron Letters,* 1981, vol. 22, 1859-1862 **[0065]**
- **Martin.** Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990 **[0069]**
- Martin, Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990, 1435-1712 **[0070]**
- **Mann CJ et al.** Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse. *Proc., Natl. Acad. Science,* 2001, vol. 98 (1), 42-47 **[0074]**
- **Gebski et al.** *Human Molecular Genetics,* 2003, vol. 12 (15), 1801-1811 **[0074]**
- **Fraley et al.** *Trends Biochem. Sci.,* 1981, vol. 6, 77 **[0077]**
- **Mannino et al.** *Biotechniques,* 1988, vol. 6, 682 **[0078]**
- **Friedmann.** *Science,* 1989, vol. 244, 1275-1280 **[0081]**
- **Rosenberg.** *Cancer Research,* 1991, vol. 51 (18), 5074S-5079S **[0082]**
- **Rosenfeld et al.** *Cell,* 1992, vol. 68, 143-155 **[0082]**
- **Rosenfeld et al.** *Science,* 1991, vol. 252, 431-434 **[0082]**
- **Brigham et al.** *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0082]**
- **Nabel et al.** *Science,* 1990, vol. 249, 1285-1288 **[0082]**
- **Hazinski et al.** *Am. J. Resp. Cell Molec. Biol.,* 1991, vol. 4, 206-209 **[0082]**
- **Wang ; Huang.** *Proc. Natl. Acad. Sci.,* 1987, vol. 84, 7851-7855 **[0082]**
- **Wu ; Wu.** *J. Biol. Chem.,* 1988, vol. 263, 14621-14624 **[0082]**
- **Wolff et al.** *Science,* 1990, vol. 247, 1465-1468 **[0082]**
- **The Brigham et al.** *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0082]**
- *Clinical Research,* 1991, 39 **[0082]**
- **Anderson.** *Science,* 1992, vol. 256, 808-813 **[0082]**
- **Sambrook et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989 **[0091]**
- DNA Cloning: A Practical Approach. MRL Press, Ltd, 1985, vol. I, II **[0091]**
- **Ausubel, F. ; Brent, R. ; Kingston, R.E. ; Moore, D.D. ; Seidman, J.G. ; Smith, J.A. ; Struhl, K.** Current Protocols In Molecular Biology. Greene Publishing Associates/Wiley Intersciences, 2002 **[0091]**

EXHIBIT AT

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 2 206 781 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention of the grant of the patent:
02.12.2015 Bulletin 2015/49

(21) Application number: **10004274.6**

(22) Date of filing: **28.06.2005**

(51) Int Cl.:
C12N 15/113 *(2010.01)*
A61K 48/00 *(2006.01)*
A61K 31/7088 *(2006.01)*
A61K 31/712 *(2006.01)*
C07H 21/00 *(2006.01)*

(54) **Antisense oligonucleotides for inducing exon skipping and methods of use thereof**

Antisense-Oligonukleotide zur Induktion von Exon-Skipping sowie Verfahren zur Verwendung davon

Oligonucléotides antisens permettant d'induire un saut d'exon et leurs procédés d'utilisation

(84) Designated Contracting States:
AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR
Designated Extension States:
HR LV

(30) Priority: **28.06.2004 AU 2004903474**

(43) Date of publication of application:
14.07.2010 Bulletin 2010/28

(60) Divisional application:
12162995.0 / 2 500 430
15168694.6 / 2 933 332
15190341.6

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
05754344.9 / 1 766 010

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(56) References cited:
WO-A1-93/20227
WO-A2-03/053341
WO-A2-2004/083446
CA-A1- 2 507 125

- **AARTSMA-RUS, A. ET AL.: "Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients", HUMAN MOLECULAR GENETICS, vol. 12, no. 8, 2003, pages 907-914, XP008084159, ISSN: 0964-6906**
- **AARTSMA-RUS A ET AL: "Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy", NEUROMUSCULAR DISORDERS,, vol. 12, 1 January 2002 (2002-01-01), pages 71-77, XP008116183, DOI: 10.1016/S0960-8966(02) 00086-X**
- **MATSUO M: "DUCHENNE AND BECKER MUSCULAR DYSTROPHY: FROM GENE DIAGNOSIS TO MOLECULAR THERAPY", IUBMB LIFE, vol. 53, no. 3, 1 March 2002 (2002-03-01), pages 147-152, XP009021242, ISSN: 1521-6543**
- **ERRINGTON STEPHEN J ET AL: "Target selection for antisense oligonucleotide induced exon skipping in the dystrophin gene.", THE JOURNAL OF GENE MEDICINE, vol. 5, no. 6, June 2003 (2003-06), pages 518-527, XP002559309, ISSN: 1099-498X**

EP 2 206 781 B1

Remarks:

- This application was filed on 22-04-2010 as a divisional application to the application mentioned under INID code 62.
- The file contains technical information submitted after the application was filed and not included in this specification

Description

Field of the Invention

[0001] The present invention relates to novel antisense compounds and compositions suitable for facilitating exon skipping. It also provides methods for inducing exon skipping using the novel antisense compounds as well as therapeutic compositions adapted for use in the methods of the invention.

Background Art

[0002] Significant effort is currently being expended researching methods for suppressing or compensating for disease-causing mutations in genes. Antisense technologies are being developed using a range of chemistries to affect gene expression at a variety of different levels (transcription, splicing, stability, translation). Much of that research has focused on the use of antisense compounds to correct or compensate for abnormal or disease-associated genes in a myriad of different conditions.

[0003] Antisense molecules are able to inhibit gene expression with exquisite specificity and because of this many research efforts concerting oligonucleotides as modulators of gene expression have focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The antisense oligonucleotides are directed either against RNA (sense strand) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II. To achieve a desired effect in specific gene down-regulation, the oligonucleotides must either promote the decay of the targeted mRNA or block translation of that mRNA, thereby effectively preventing *de novo* synthesis of the undesirable target protein.

[0004] Such techniques are not useful where the object is to up-regulate production of the native protein or compensate for mutations which induce premature termination of translation such as nonsense or frame-shifting mutations. Furthermore, in cases where a normally functional protein is prematurely terminated because of mutations therein, a means for restoring some functional protein production through antisense technology has been shown to be possible through intervention during the splicing processes (Sierakowska H, et al., (1996) Proc Natl Acad Sci USA 93, 12840-12844; Wilton SD, et al., (1999) Neuromusc Disorders 9, 330-338; van Deutekom JC et al., (2001) Human Mol Genet 10, 1547-1554). In these cases, the defective gene transcript should not be subjected to targeted degradation so the antisense oligonucleotide chemistry should not promote target mRNA decay.

[0005] In a variety of genetic diseases, the effects of mutations on the eventual expression of a gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules. It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanism invoked have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts.

[0006] In nature, the extent of genetic deletion or exon skipping in the splicing process is not fully understood, although many instances have been documented to occur, generally at very low levels (Sherrat TG, et al., (1993) Am J Hum Genet 53, 1007-1015). However, it is recognised that if exons associated with disease-causing mutations can be specifically deleted from some genes, a shortened protein product can sometimes be produced that has similar biological properties of the native protein or has sufficient biological activity to ameliorate the disease caused by mutations associated with the target exon (Lu QL, et al., (2003) Nature Medicine 9, 1009-1014; Aartsma-Rus A et al., (2004) Am J Hum Genet 74: 83-92).

[0007] This process of targeted exon skipping is likely to be particularly useful in long genes where there are many exons and introns, where there is redundancy in the genetic constitution of the exons or where a protein is able to function without one or more particular exons (e.g. with the dystrophin gene, which consists of 79 exons; or possibly some collagen genes which encode for repeated blocks of sequence or the huge nebulin or titin genes which are comprised of ~80 and over 370 exons, respectively).

[0008] Efforts to redirect gene processing for the treatment of genetic diseases associated with truncations caused by mutations in various genes have focused on the use of antisense oligonucleotides that either (1) fully or partially overlap with the elements involved in the splicing process; or (2) bind to the pre-mRNA at a position sufficiently close to the element to disrupt the binding and function of the splicing factors that would normally mediate a particular splicing reaction which occurs at that element (e.g., binds to the pre-mRNA at a position within 3, 6, or 9 nucleotides of the

element to be blocked).

[0009] For example, modulation of mutant dystrophin pre-mRNA splicing with antisense oligoribonucleotides has been reported both *in vitro* and *in vivo*. In one type of dystrophin mutation reported in Japan, a 52-base pair deletion mutation causes exon 19 to be removed with the flanking introns during the splicing process (Matsuo et al., (1991) J Clin Invest. 87:2127-2131). An *in vitro* minigene splicing system has been used to show that a 31-mer 2'-O-methyl oligoribonucleotide complementary to the 5' half of the deleted sequence in dystrophin Kobe exon 19 inhibited splicing of wild-type *pre-mRNA* (Takeshima et al. (1995), J. Clin. Invest., 95, 515-520). The same oligonucleotide was used to induce exon skipping from the native dystrophin gene transcript in human cultured lymphoblastoid cells.

[0010] Dunckley et al., (1997) Nucleosides & Nucleotides, 16, 1665-1668 described *in vitro* constructs for analysis of splicing around exon 23 of mutated dystrophin in the *mdx* mouse mutant, a model for muscular dystrophy. Plans to analyse these constructs *in vitro* using 2' modified oligonucleotides targeted to splice sites within and adjacent to mouse dystrophin exon 23 were discussed, though no target sites or sequences were given.

[0011] 2'-O-methyl oligoribonucleotides were subsequently reported to correct dystrophin deficiency in myoblasts from the *mdx* mouse from this group. An antisense oligonucleotide targeted to the 3' splice site of murine dystrophin intron 22 was reported to cause skipping of the mutant exon as well as several flanking exons and created a novel in-frame dystrophin transcript with a novel internal deletion. This mutated dystrophin was expressed in 1-2% of antisense treated mdx myotubes. Use of other oligonucleotide modifications such as 2'-O-methoxyethyl phosphodiesters are described (Dunckley et al. (1998) Human Mol. Genetics, 5, 1083-90).

[0012] Thus, antisense molecules may provide a tool in the treatment of genetic disorders such as Duchenne Muscular Dystrophy (DMD). However, attempts to induce exon skipping using antisense molecules have had mixed success. Studies on dystrophin exon 19, where successful skipping of that exon from the dystrophin pre-mRNA was achieved using a variety of antisense molecules directed at the flanking splice sites or motifs within the exon involved in exon definition as described by Errington et al. (2003) J Gen Med 5, 518-527".

[0013] In contrast to the apparent ease of exon 19 skipping, the first report of exon 23 skipping in the *mdx* mouse by Dunckley *et al*., (1998) is now considered to be reporting only a naturally occurring revertant transcript or artefact rather than any true antisense activity. In addition to not consistently generating transcripts missing exon 23, Durickley *et al*., (1998) did not show any time course of induced exon skipping, or even titration of antisense oligonucleotides, to demonstrate dose dependent effects where the levels of exon skipping corresponded with increasing or decreasing amounts of antisense oligonucleotide. Furthermore, this work could not be replicated by other researchers.

[0014] The first example of specific and reproducible exon skipping in the *mdx* mouse model was reported by Wilton et al., (1999) Neuromuscular Disorders 9, 330-338. By directing an antisense molecule to the donor splice site, consistent and efficient exon 23 skipping was induced in the dystrophin mRNA within 6 hours of treatment of the cultured cells. Wilton *et al*., (1999), also describe targeting the acceptor-region of the mouse dystrophin pre-mRNA with longer antisense oligonucleotides and being unable to repeat the published results of Dunckley *et al*., (1998). No exon skipping, either 23 alone or multiple removal of several flanking exons, could be reproducibly detected using a selection of antisense oligonucleotides directed at the acceptor splice site of intron 22.

[0015] While the first antisense oligonucleotide directed at the intron 23 donor splice site induced consistent exon skipping in primary cultured myoblasts, this compound was found to be much less efficient in immortalized cell cultures expressing higher levels of dystrophin. However, with refined targeting and antisense oligonucleotide design, the efficiency of specific exon removal was increased by almost an order of magnitude (see Mann CJ et al., (2002) J Gen Med 4, 644-654).

[0016] Other disclosures relating to DMD therapy include CA 2507125, Aartsma-Rus et al., Human Molecular Genetics 12, (2003) 907-14, Aartsma-rus et al., Neuromuscular Disorders, 12 (2002) 71-7, as well as WO 2004/083446, published on 30 September 2004.

[0017] Thus, there remains a need to provide antisense oligonucleotides capable of binding to and modifying the splicing of a target nucleotide sequence. Simply directing the antisense oligonucleotides to motifs presumed to be crucial for splicing is no guarantee of the efficacy of that compound in a therapeutic setting.

Summary of the Invention

[0018] The present invention provides an isolated antisense oligonucleotide that binds to human dystrophin pre-mRNA, wherein said oligonucleotide is 20 to 31 nucleotides in length and is an oligonucleotide that is specifically hybridizable to an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47), annealing site H53A (+39+69), or both, wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide, and, wherein said oligonucleotide induces exon 53 skipping..

[0019] The invention further provides a composition comprising an antisense oligonucleotide according to the invention and a saline solution that includes a phosphate buffer.

[0020] The invention further provides an antisense oligonucleotide according to the invention, or a composition ac-

cording to the invention, for use in a method of treatment of muscular dystrophy.

[0021] The invention is further defined in the accompanying claims.

[0022] The choice of target selection plays a crucial role in the efficiency of exon skipping and hence its subsequent application of a potential therapy. Simply designing antisense molecules to target regions of pre-mRNA presumed to be involved in splicing is no guarantee of inducing efficient and specific exon skipping. The most obvious or readily defined targets for splicing intervention are the donor and acceptor splice sites although there are less defined or conserved motifs including exonic splicing enhancers, silencing elements and branch points.

[0023] The acceptor and donor splice sites have consensus sequences of about 16 and 8 bases respectively (see Figure 1 for schematic representation of motifs and domains involved in exon recognition, intron removal and the splicing process).

[0024] The invention also addresses the use of purified and isolated antisense oligonucleotides of the invention, for the manufacture of a medicament for treatment of a genetic disease.

[0025] The invention may be used for treating a condition characterised by Duchenne muscular dystrophy, by administering to a patient in need of treatment an effective amount of an appropriately designed antisense oligonucleotide of the Invention, relevant to the particular genetic lesion in that patient.

[0026] Further, the invention may be used for prophylactically treating a patient to prevent or at least minimize Duchenne muscular dystrophy, comprising the step of: administering to the patient an effective amount of an antisense oligonucleotide or a pharmaceutical composition comprising one or more of these biological molecules.

[0027] Also described herein are kits for treating a genetic disease, which kits comprise at least a antisense oligonucleotide of the present invention, packaged in a suitable container and instructions for its use.

[0028] Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following figures.

Brief Description of the Drawings

[0029]

Figure 1 Schematic representation of motifs and domains Involved in exon recognition, intron removal and the splicing process.

Figure 2 Diagrammatic representation of the concept of antisense oligonucleotide induced exon skipping to by-pass disease-causing mutations (not drawn to scale). The hatched box represents an exon carrying a mutation that prevents the translation of the rest of the mRNA into a protein. The solid black bar represents an antisense oligonucleotide that prevents inclusion of that exon in the mature mRNA.

Figure 3 Gel electrophoresis showing exon 51, exon 52 and exon 53 skipping using various antisense molecules directed at exons 51, 52 and 53, respectively. A "cocktail" of antisense molecules is also shown directed at exon 53

Brief Description of the Sequence Listings

[0030]

Table 1: Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophie pre-mRNA. Since these 2'-O-methyl antisense oligonucleotides are more RNA-like, U represents uracil. With other antisense chemistries such as peptide nucleic acids or morpholinos, these U bases may be shown as **"T"**.

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5'-3')
192	H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG
193	H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G
195	H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C

Detailed Description of the Invention

General

[0031] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variation and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0032] The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally equivalent products, compositions and methods are clearly within the scope of the invention as described herein.

[0033] Sequence identity numbers (SEQ ID NO:) containing nucleotide and amino acid sequence information included in this specification are collected at the end of the description and have been prepared using the programme PatentIn Version 3.0. Each nucleotide or amino acid sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, etc.). The length, type of sequence and source organism for each nucleotide or amino acid sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide and amino acid sequences referred to in the specification are defined by the information provided in numeric indicator field <400> followed by the sequence identifier (e.g. <400>1, <400>2, etc.).

[0034] An antisense molecules nomenclature system was proposed and published to distinguish between the different antisense molecules (see Mann et al., (2002) J Gen Med 4, 644-654). This nomenclature became especially relevant when testing several slightly different antisense molecules, all directed at the same target region, as shown below:

$$H \# A/D (x : y).$$

[0035] The first letter designates the species (e.g. H: human, M: murine, C: canine)

[0036] "#" designates target dystrophin exon number.

[0037] "A/D" indicates acceptor or donor splice site at the beginning and end of the exon, respectively.

[0038] (x y) represents the annealing coordinates where "-" or "+" indicate intronic or exonic sequences respectively. As an example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest splice site would be the acceptor so these coordinates would be preceded with an "A". Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by A(+65+85), that is the site between the 65th and 85th nucleotide from the start of that exon.

[0039] No admission is made that any of the references constitute prior art or are part of the common general knowledge of those working in the field to which this invention relates.

[0040] As used necessarily herein the term "derived" and "derived from" shall be taken to indicate that a specific integer may be obtained from a particular source *albeit* not directly from that source.

[0041] Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

[0042] Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

Description of the Preferred Embodiment

[0043] When antisense molecule(s) are targeted to nucleotide sequences involved in splicing in exons within pre-mRNA sequences, normal splicing of the exon may be inhibited causing the splicing machinery to by-pass the entire mutated exons from the mature mRNA. The concept of antisense oligonucleotide induced exon skipping is shown in Figure 2. In many genes, deletion of an entire exon would lead to the production of a non-functional protein through the loss of important functional domains or the disruption of the reading frame. In some proteins, however, it is possible to shorten the protein by deleting one or more exons, without disrupting the reading frame, from within the protein without seriously altering the biological activity of the protein. Typically, such proteins have a structural role and or possess functional domains at their ends. The present invention describes antisense molecules capable of binding to specified dystrophin pre-mRNA targets and re-directing processing of that gene.

Antisense Molecules

[0044] According to a first aspect of the invention, there is provided antisense molecules as defined in the claims capable of binding to a selected target to induce exon skipping. To induce exon skipping in exons of the Dystrophin gene transcript, the antisense molecules are preferably selected from the group of compounds shown in Table 1. Also described is a combination or "cocktail" of two or more antisense oligonucleotides capable of binding to a selected target to induce exon skipping.

[0045] Designing antisense molecules to completely mask consensus splice sites may not necessarily generate any skipping of the targeted exon. Furthermore, the inventors have discovered that size or length of the antisense oligonucleotide itself is not always a primary factor when designing antisense molecules. With some targets such as exon 19, antisense oligonucleotides as short as 12 bases were able to induce exon skipping, albeit not as efficiently as longer (20-31 bases) oligonucleotides. In some other targets, such as murine dystrophin exon 23, antisense oligonucleotides only 17 residues long were able to induce more efficient skipping than another overlapping compound of 25 nucleotides.

[0046] The inventors have also discovered that there does not appear to be any standard motif that can be blocked or masked by antisense molecules to redirect splicing. In some exons, such as mouse dystrophin exon 23, the donor splice site was the most amenable to target to re-direct skipping of that exon. It should be noted that designing and testing a series of exon 23 specific antisense molecules to anneal to overlapping regions of the donor splice site showed considerable variation in the efficacy of induced exon skipping. As reported in Mann et al., (2002) there was a significant variation in the efficiency of bypassing the nonsense mutation depending upon antisense oligonucleotide annealing ("Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy". J Gen Med 4: 644-654). Targeting the acceptor site of exon 23 or several internal domains was not found to induce any consistent exon 23 skipping.

[0047] In other exons targeted for removal, masking the donor splice site did not induce any exon skipping. However, by directing antisense molecules to the acceptor splice site (human exon 8), strong and sustained exon skipping was induced. It should be noted that removal of human exon 8 was tightly linked with the co-removal of exon 9. There is no strong sequence homology between the exon 8 antisense oligonucleotides and corresponding regions of exon 9 so it does not appear to be a matter of cross reaction. Rather the splicing of these two exons is inextricably linked. This is not an isolated instance as the same effect is observed in canine cells where targeting exon 8 for removal also resulted in the skipping of exon 9. Targeting exon 23 for removal in the mouse dystrophin pre-mRNA also results in the frequent removal of exon 22 as well. This effect occurs in a dose dependent manner and also indicates close coordinated processing of 2 adjacent exons.

[0048] In other targeted exons, antisense molecules directed at the donor or acceptor splice sites did not induce exon skipping while annealing antisense molecules to intra-exonic regions (i.e. exon splicing enhancers within human dystrophin exon 6) was most efficient at inducing exon skipping. Some exons, both mouse and human exon 19 for example, are readily skipped by targeting antisense molecules to a variety of motifs. That is, targeted exon skipping is induced after using antisense oligonucleotides to mask donor and acceptor splice sites or exon splicing enhancers.

[0049] To identify and select antisense oligonucleotides suitable for use in the modulation of exon skipping, a nucleic acid sequence whose function is to be modulated must first be identified. This may be, for example, a gene (or mRNA transcribed form the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites, or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0050] Preferably, the present invention aims to provide antisense molecules capable of binding to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping. Duchenne muscular dystrophy arises from mutations that preclude the synthesis of a functional dystrophin gene product. These Duchenne muscular dystrophy gene defects are typically nonsense mutations or genomic rearrangements such as deletions, duplications or micro-deletions or insertions that disrupt the reading frame. As the human dystrophin gene is a large and complex gene with the 79 exons being spliced together to generate a mature mRNA with an open reading frame of approximately 11,000 bases, there are many positions where these mutations can occur. Consequently, a comprehensive antisense oligonucleotide based therapy to address many of the different disease-causing mutations in the dystrophin gene will require that many exons can be targeted for removal during the splicing process.

[0051] Within the context of the present invention, preferred target site(s) are those involved in mRNA splicing (i.e. splice donor sites, splice acceptor sites or exonic splicing enhancer elements). Splicing branch points and exon recognition sequences or splice enhancers are also potential target sites for modulation of mRNA splicing.

[0052] The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridisable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA

target.

[0053] It is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable. An antisense molecule is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

[0054] While the above method may be used to select antisense molecules capable of deleting any exon from within a protein that is capable of being shortened without affecting its biological function, the exon deletion should not lead to a reading frame shift in the shortened transcribed mRNA. Thus, if in a linear sequence of three exons the end of the first exon encodes two of three nucleotides in a codon and the next exon is deleted then the third exon in the linear sequence must start with a single nucleotide that is capable of completing the nucleotide triplet for a codon. If the third exon does not commence with a single nucleotide there will be a reading frame shift that would lead to the generation of truncated or a non-functional protein.

[0055] It will be appreciated that the codon arrangements at the end of exons in structural proteins may not always break at the end of a codon, consequently there may be a need to delete more than one exon from the pre-mRNA to ensure in-frame reading of the mRNA. In such circumstances, a plurality of antisense oligonucleotides may need to be selected by the method of the invention wherein each is directed to a different region responsible for inducing splicing in the exons that are to be deleted.

[0056] The length of an antisense molecule may vary so long as it is capable of binding selectively to the intended location within the pre-mRNA molecule. The length of such sequences can be determined in accordance with selection procedures described herein. Generally, the antisense molecule will be from about 10 nucleotides in length up to about 50 nucleotides in length. It will be appreciated however that any length of nucleotides within this range may be used in the method. Preferably, the length of the antisense molecule is between 17 to 30 nucleotides in length.

[0057] In order to determine which exons can be connected in a dystrophin gene, reference should be made to an exon boundary map. Connection of one exon with another is based on the exons possessing the same number at the 3' border as is present at the 5' border of the exon to which it is being connected. Therefore, if exon 7 were deleted, exon 6 must connect to either exons 12 or 18 to maintain the reading frame. Thus, antisense oligonucleotides would need to be selected which redirected splicing for exons 7 to 11 in the first instance or exons 7 to 17 in the second instance. Another and somewhat simpler approach to restore the reading frame around an exon 7 deletion would be to remove the two flanking exons. Induction of exons 6 and 8 skipping should result in an in-frame transcript with the splicing of exons 5 to 9. In practise however, targeting exon 8 for removal from the pre-mRNA results in the co-removal of exon 9 so the resultant transcript would have exon 5 joined to exon 10. The inclusion or exclusion of exon 9 does not alter the reading frame. Once the antisense molecules to be tested have been identified, they are prepared according to standard techniques known in the art. The most common method for producing antisense molecules is the methylation of the 2' hydroxyribose position and the incorporation of a phosphorothioate backbone produces molecules that superficially resemble RNA but that are much more resistant to nuclease degradation.

[0058] To avoid degradation of pre-mRNA during duplex formation with the antisense molecules, the antisense molecules used in the method may be adapted to minimise or prevent cleavage by endogenous RNase H. This property is highly preferred as the treatment of the RNA with the unmethylated oligonucleotides either intracellularly or in crude extracts that contain RNase H leads to degradation of the pre-mRNA: antisense oligonucleotide duplexes. Any form of modified antisense molecules that is capable of by-passing or not inducing such degradation may be used in the present method. An example, of antisense molecules which when duplexed with RNA are not cleaved by cellular RNase H is 2'-O-methyl derivatives. 2'-O-methyl-oligoribonucleotides are very stable in a cellular environment and in animal tissues, and their duplexes with RNA have higher Tm values than their ribo- or deoxyribo- counterparts.

[0059] Antisense molecules that do not activate RNase H can be made in accordance with known techniques (see, e.g., U.S. Pat. 5,149,797). Such antisense molecules, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the portions of the oligonucleotide involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense molecules that do not activate RNase H are available. For example, such antisense molecules may be oligonucleotides wherein at least one, or all, of the inter-nucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another nonlimiting example, such antisense molecules are molecules wherein at least one, or all, of the nucleotides contain a 2' lower alkyl moiety (e.g., C_1-C_4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl,

and isopropyl). For example, every other one of the nucleotides may be modified as described.

[0060] While antisense oligonucleotides are a preferred form of the antisense molecules, the present invention comprehends other oligomeric antisense molecules, including but not limited to oligonucleotide mimetics such as are described below.

[0061] Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural inter-nucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom In their inter-nucleoside backbone can also be considered to be oligonucleosides.

[0062] In other preferred oligonucleotide mimetics, both the sugar and the inter-nucleoside linkage, i.e., the backbone; of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleo-bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

[0063] Modified oligonucleotides may also contain one or more substituted sugar moieties. Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. Certain nucleo-bases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These Include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

[0064] Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-S-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

[0065] It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds that are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense molecules, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the Increased resistance to nuclease degradation, increased cellular uptake, and an additional region for increased binding affinity for the target nucleic acid.

Methods of Manufacturing Antisense Molecules

[0066] The antisense molecules used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). One method for synthesising oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066.

[0067] Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. In one such automated embodiment, diethyl-phosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al., (1981) Tetrahedron Letters, 22:1859-1862.

[0068] The antisense molecules of the invention are synthesised *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules. The molecules of the invention may also be mixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

Therapeutic Agents

[0069] The present invention also can be used as a prophylactic or therapeutic, which may be utilised for the purpose

of treatment of a genetic disease.

[0070] Accordingly, in one embodiment the present invention provides antisense molecules that bind to a selected target in the dystrophin pre-mRNA to induce efficient and consistent exon skipping described herein in a therapeutically effective amount admixed with a pharmaceutically acceptable carrier, diluent, or excipient.

[0071] The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to a patient. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solution. Suitable pharmaceutical carriers are described in Martin, Remington's Pharmaceutical Sciences, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

[0072] In a more specific form of the invention there are provided pharmaceutical compositions comprising therapeutically effective amounts of an antisense molecule together with pharmaceutically acceptable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. Such compositions include diluents of various buffer content (*e.g.*, Tris-HCl, acetate, phosphate), pH and ionic strength and additives such as detergents and solubilizing agents (e.g., Tween 80, Polysorbate 80), anti-oxidants (*e.g.*, ascorbic acid, sodium metabisulfite), preservatives (*e.g.,* Thimersol, benzyl alcohol) and bulking substances (*e.g.,* lactose, mannitol). The material may be incorporated into particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, etc. or into liposomes. Hylauronic acid may also be used. Such compositions may influence the physical state, stability, rate of *in vivo* release, and rate of *in vivo* clearance of the present proteins and derivatives. *See, e.g.,* Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing Co., Easton, PA 18042) pages 1435-1712. The compositions may be prepared in liquid form, or may be in dried powder, such as lyophilised form.

[0073] It will be appreciated that pharmaceutical compositions provided according to the present invention may be administered by any means known in the art. Preferably, the pharmaceutical compositions for administration are administered by injection, orally, or by the pulmonary, or nasal route. The antisense molecules are more preferably delivered by intravenous, intra-arterial, intraperitoneal, intramuscular, or subcutaneous routes of administration.

<u>Antisense molecule based therapy</u>

[0074] Also addressed by the present invention is the use of antisense molecules of the present invention, for manufacture of a medicament for modulation of a genetic disease.

[0075] The delivery of a therapeutically useful amount of antisense molecules may be achieved by methods previously published. For example, intracellular delivery of the antisense molecule may be via a composition comprising an admixture of the antisense molecule and an effective amount of a block copolymer. An example of this method is described in US patent application US 20040248833.

[0076] Other methods of delivery of antisense molecules to the nucleus are described in Mann CJ et al., (2001) ["Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse". Proc., Natl. Acad. Science, 98(1) 42-47] and in Gebski et al., (2003). Human Molecular Genetics, 12(15): 1801-1811.

[0077] A method for introducing a nucleic acid molecule into a cell by way of an expression vector either as naked DNA or complexed to lipid carriers, is described in US patent US 6,806,084.

[0078] It may be desirable to deliver the antisense molecule in a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes or liposome formulations.

[0079] Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. These formulations may have net cationic, anionic or neutral charge characteristics and are useful characteristics with *in vitro, in vivo* and *ex vivo* delivery methods. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0 .PHI.m can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, and DNA can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley, et al., Trends Biochem. Sci., 6:77, 1981).

[0080] In order for a liposome to be an efficient gene transfer vehicle, the following characteristics should be present: (1) encapsulation of the antisense molecule of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, et al., Biotechniques, 6:682, 1988).

[0081] The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of

divalent cations.

[0082] Alternatively, the antisense construct may be combined with other pharmaceutically acceptable carriers or diluents to produce a pharmaceutical composition. Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

[0083] The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition. Multiple approaches for introducing functional new genetic material into cells, both in vitro and in vivo have been attempted (Friedmann (1989) Science, 244:1275-1280).

[0084] These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) supra; Rosenberg (1991) Cancer Research 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors (Rosenfeld, et al. (1992) Cell, 68:143-155; Rosenfeld, et al. (1991) Science, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), supra; Brigham, et al. (1989) Am. J. Med. Sci.. 298:278-281; Nabel, et al. (1990) Science, 249:1285-1288; Hazinski, et al. (1991) Am. J. Resp. Cell Molec. Biol., 4:206-209; and Wang and Huang (1987) Proc. Natl. Acad. Sci. (USA), 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) J. Biol. Chem., 263:14621-14624) or the use of naked DNA, expression vectors (Nabel et al. (1990), supra); wolff et al. (1990) Science, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) supra); Rosenfeld et al. (1991) supra; Brigham et al. (1989) supra; Nabel (1990) supra; and Hazinski et al. (1991) supra). The Brigham et al. group (Am. J. Med. Sci. (1989) 298:278-281 and Clinical Research (1991) 39 (abstract)) have reported in vivo transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, Science (1992) 256:808-813.

[0085] The antisense molecules of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such pro-drugs, and other bioequivalents.

[0086] The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

[0087] For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, (including by nebulizer, intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

[0088] The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Kits of the Invention

[0089] The invention also provides kits for treatment of a patient with a genetic disease which kit comprises at least an antisense molecule, packaged in a suitable container, together with instructions for its use.

[0090] In a preferred embodiment, the kits will contain at least one antisense molecule as defined in the claims. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

[0091] Those of ordinary skill in the field should appreciate that applications of the above method has wide application

for identifying antisense molecules suitable for use in the treatment of many other diseases.

EXAMPLES

[0092] The following Examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these Examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

[0093] Methods of molecular cloning, immunology and protein chemistry, which are not explicitly described in the following examples, are reported in the literature and are known by those skilled in the art. General texts that described conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art, included, for example: Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989); Glover ed., DNA Cloning: A Practical Approach, Volumes I and II, MRL Press, Ltd., Oxford, U.K. (1985); and Ausubel, F., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Intersciences, New York (2002).

Determining Induced Exon Skipping in Human Muscle Cells

[0094] Attempts by the inventors to develop a rational approach in antisense molecules design were not completely successful as there did not appear to be a consistent trend that could be applied to all exons. As such, the identification of the most effective and therefore most therapeutic antisense molecules compounds has been the result of empirical studies.

[0095] These empirical studies involved the use of computer programs to identify motifs potentially involved in the splicing process. Other computer programs were also used to identify regions of the pre-mRNA which may not have had extensive secondary structure and therefore potential sites for annealing of antisense molecules. Neither of these approaches proved completely reliable in designing antisense oligonucleotides for reliable and efficient induction of exon skipping.

[0096] Annealing sites on the human dystrophin pre-mRNA were selected for examination, initially based upon known or predicted motifs or regions involved in splicing. 2OMe antisense oligonucleotides were designed to be complementary to the target sequences under investigation and were synthesised on an Expedite 8909 Nucleic Acid Synthesiser. Upon completion of synthesis, the oligonucleotides were cleaved from the support column and de-protected in ammonium hydroxide before being desalted. The quality of the oligonucleotide synthesis was monitored by the intensity of the trityl signals upon each deprotection step during the synthesis as detected in the synthesis log. The concentration of the antisense oligonucleotide was estimated by measuring the absorbance of a diluted aliquot at 260nm.

[0097] Specified amounts of the antisense molecules were then tested for their ability to induce exon skipping in an *in vitro* assay, as described below.

[0098] Briefly, normal primary myoblast cultures were prepared from human muscle biopsies obtained after informed consent. The cells were propagated and allowed to differentiate into myotubes using standard culturing techniques. The cells were then transfected with the antisense oligonucleotides by delivery of the oligonucleotides to the cells as cationic lipoplexes, mixtures of antisense molecules or cationic liposome preparations.

[0099] The cells were then allowed to grow for another 24 hours, after which total RNA was extracted and molecular analysis commenced. Reverse transcriptase amplification (RT-PCR) was undertaken to study the targeted regions of the dystrophin pre-mRNA or induced exonic re-arrangements.

[0100] For example, in the testing of an antisense molecule for inducing exon 19 skipping the RT-PCR test scanned several exons to detect involvement of any adjacent exons. For example, when inducing skipping of exon 19, RT-PCR was carried out with primers that amplified across exons 17 and 21. Amplifications of even larger products in this area (i.e. exons 13-26) were also carried out to ensure that there was minimal amplification bias for the shorter induced skipped transcript. Shorter or exon skipped products tend to be amplified more efficiently and may bias the estimated of the normal and induced transcript.

[0101] The sizes of the amplification reaction products were estimated on an agarose gel and compared against appropriate size standards. The final confirmation of identity of these products was carried out by direct DNA sequencing to establish that the correct or expected exon junctions have been maintained.

[0102] Once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the assay as described above. Our definition of an efficient antisense molecule is one that induces strong and sustained exon skipping at transfection concentrations in the order of 300 nM or less.

Antisense Oligonucleotides Directed at Exon 53

[0103] Antisense oligonucleotides directed at exon 53 were prepared and tested for their ability to induce exon skipping in human muscle cells using similar methods as described above.

[0104] Figure 3 also shows antisense molecule H53A(+39+69) [SEQ ID NO:193] directed at exon 53 acceptor splice site. This antisense oligonucleotide was able to induce exon 53 skipping at 5, 100, 300 and 600 nM. A "cocktail" of three exon 53 antisense oligonucleotides:- H53D(+23+47) [SEQ ID NO:195], H53A(+150+175) [SEQ ID NO:196] and H53A(+14-07) [SEQ ID NO:194], were also tested, as shown in Figure 3 and exhibited an ability to induce exon skipping.

[0105] Table 2 below includes other antisense molecules tested at a concentration range of 50, 100, 300 and 600 nM. These antisense molecules showed varying ability to induce exon 53 skipping. Antisense molecule H53A(+39+69) [SEQ ID NO:193] induced the strongest exon 53 skipping.

Table 2

Antisense oligonucleotide name	Sequence	Ability to induce skipping
H53A(+45+69)	CAU UCA ACU GUU GCC UCC GGU UCU G	Faint skipping at 50 nM
H53A(+39+62)	CUG UUG CCU CCG GUU CUG AAG GUG	Faint skipping at 50 nM
H53A(+39+69)	CAU UCA ACU GUU GCC UCC GGU UCU GAA GGU G	Strong skipping to 50 nM
H53D(+14-07)	UAC UAA CCU UGG UUU CUG UGA	Very faint skipping to 50 nM
H53A(+23+47)	CUG AAG GUG UUC UUG UAC UUC AUC C	Very faint skipping to 50 nM
H53A(+150+176)	UGU AUA GGG ACC CUC CUU CCA UGA CUC	Very faint skipping to 50 nM
H53D(+20-05)	CUA ACC UUG GUU UCU GUG AUU UUC U	Not made yet
H53D(+09-18)	GGU AUC UUU GAU ACU AAC CUU GGU UUC	Faint at 600 nM
H53A(-12+10)	AUU CUU UCA ACU AGA AUA AAA G	No skipping
H53A(-07+18)	GAU UCU GAA UUC UUU CAA CUA GAA U	No skipping
H53A(+07+26)	AUC CCA CUG AUU CUG AAU UC	No skipping
H53A(+124+145)	UUG GCU CUG GCC UGU CCU AAG A	No skipping

[0106] The invention is defined with reference to the following clauses:

Clause 1: An antisense molecule capable of binding to a selected target site to induce exon skipping in the dystrophin gene, as set forth in SEQ ID NO: 1 to 202.

Clause 2: An antisense molecule according to clause 1 capable of inducing exon skipping in exons 3, 4, 8, 10 to 16, 19 to 40, 42 to 44, 46, 47 and 50 to 53 of the dystrophin gene.

Clause 3: A combination of two or more antisense molecules according to clause 1 or clause 2 capable of binding to a selected target to induce exon skipping in the dystrophin gene.

Clause 4: A combination or two or more antisense molecules according to clause 3 selected from Table 1B.

Clause 5: A combination of two or more antisense molecules according to clause 1 or clause 2 joined together to form a "weasel", wherein said weasel is capable of binding to a selected target to induce exon skipping in the dystrophin gene.

Clause 6: A combination of two or more antisense molecules according to clause 5 selected from Table 1C.

Clause 7: The antisense molecule according to anyone of clauses 1 to 6, capable of binding to a selected target site, wherein the target site is an mRNA splicing site selected from a splicer donor site, splice acceptor sites or exonic splicing enhancer elements.

Clause 8: A method of treating muscular dystrophy in a patient comprising administering to the patient a composition comprising an antisense molecule according to anyone of clauses 1 to 6.

Clause 9: A pharmaceutical or therapeutic composition for the treatment of muscular dystrophy in a patient comprising (a) at least an antisense molecule according to anyone of clause 1 to 6, and (b) one or more pharmaceutically acceptable carriers and/or diluents.

Clause 10: The composition according to clause 9, comprising about 20 nM to 600 nM of the antisense molecule.

Clause 11. The use of an antisense molecule according to anyone of clauses 1 to 6 for the manufacture of a medicament for modulation of muscular dystrophy.

Clause 12. An antisense molecule according to anyone of clauses 1 to 6 for use in antisense molecule based therapy.

Clause 13. An antisense molecule according to anyone of clauses 1 to 6 as herein before described with reference to the examples.

Clause 14. A kit comprising at least one antisense molecule according to anyone of clauses 1 to 6, a suitable carrier and instructions for its use.

SEQUENCE LISTING

[0107]

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Claims

1. An isolated antisense oligonucleotide that binds to human dystrophin pre-mRNA wherein said oligonucleotide is 20 to 31 nucleotides in length and is an oligonucleotide that is specifically hybridizable to an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47), annealing site H53A (+39+69), or both,

wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide, and,
wherein said oligonucleotide induces exon 53 skipping.

2. An antisense oligonucleotide according to claim 1 selected from SEQ ID NOS: 192, 193 and 195, optionally wherein the uracil bases (U) are thymine bases (T).

3. The antisense oligonucleotide of claim 1 or 2, wherein the oligonucleotide is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

4. The antisense oligonucleotide of claim 3, wherein the oligonucleotide is chemically linked to a polyethylene glycol chain.

5. The antisense oligonucleotide of claim 1 or 2 comprising a 5-substituted pyrimidine base.

6. The antisense oligonucleotide of claim 1 or 2 comprising a 5-methylcytosine base.

7. A composition, comprising an antisense oligonucleotide of any one of claims 1-6 and a saline solution that includes a phosphate buffer.

8. An antisense oligonucleotide of any one of claims 1-6, or a composition of claim 7, for use in a method of treatment of muscular dystrophy.

9. The antisense oligonucleotide or composition for use according to claim 8, wherein the muscular dystrophy is Duchenne Muscular Dystrophy.

Patentansprüche

1. Isoliertes Antisense-Oligonucleotid, das an menschliche Dystrophin-Prä-mRNA bindet, worin das Oligonucleotid 20 bis 31 Nucleotide lang ist und ein Oligonucleotid ist, das spezifisch an eine Exon-53-Target-Region des Dystrophin-Gens hybridisierbar ist, die als Annealing-Stelle H53A (+23+47), Annealing-Stelle H53A (+39+69) oder beides bezeichnet wird,
worin das Antisense-Oligonucleotid ein Morpholino-Antisense-Oligonucleotid ist und
worin das Oligonucleotid ein Überspringen von Exon 53 induziert.

2. Antisense-Oligonucleotid nach Anspruch 1, ausgewählt aus den SEQ-ID Nr. 192, 193 und 195, worin die Uracil-Basen (U) gegebenenfalls Thymin-Basen (T) sind.

3. Antisense-Oligonucleotid nach Anspruch 1 oder 2, worin das Oligonucleotid chemisch an eine oder mehrere Gruppierungen oder Konjugate gebunden ist, die die Aktivität, Zellverteilung oder Zellaufnahme des Antisense-Oligonucleotids verbessern.

4. Antisense-Oligonucleotid nach Anspruch 3, worin das Oligonucleotid chemisch an eine Polyethylenglykol-Kette gebunden ist.

5. Antisense-Oligonucleotid nach Anspruch 1 oder 2, umfassend eine 5-substituierte Pyrimidin-Base.

6. Antisense-Oligonucleotid nach Anspruch 1 oder 2, umfassend eine 5-Methylcytosin-Base.

7. Zusammensetzung, umfassend ein Antisense-Oligonucleotid nach einem der Ansprüche 1-6 und eine Kochsalzlösung, die einen Phosphatpuffer umfasst.

8. Antisense-Oligonucleotid nach einem der Ansprüche 1-6 oder Zusammensetzung nach Anspruch 7 zur Verwendung in einem Verfahren zur Behandlung von Muskeldystrophie.

9. Antisense-Oligonucleotid oder Zusammensetzung zur Verwendung nach Anspruch 8, worin die Muskeldystrophie eine Muskeldystrophie Duchenne ist.

Revendications

1. Oligonucléotide antisens isolé qui se lie à un pré-ARNm de la dystrophine humaine, où ledit oligonucléotide possède une longueur de 20 à 31 nucléotides et est un oligonucléotide qui peut spécifiquement s'hybrider à une région cible de l'exon 53 du gène de la dystrophine désignée en tant que site d'annelage H53A (+23+47), site d'annelage H53A (+39+69), ou les deux,
où ledit oligonucléotide antisens est un oligonucléotide antisens morpholino, et,
où ledit oligonucléotide induit un saut de l'exon 53.

2. Oligonucléotide antisens selon la revendication 1 sélectionné parmi SEQ ID NO: 192, 193 et 195, facultativement où les bases uracile (U) sont des bases thymine (T).

3. Oligonucléotide antisens selon la revendication 1 ou 2, où l'oligonucléotide est chimiquement lié à un ou plusieurs fragments ou conjugués qui améliorent l'activité, la distribution cellulaire, ou l'absorption cellulaire de l'oligonucléotide antisens.

4. Oligonucléotide antisens selon la revendication 3, où l'oligonucléotide est chimiquement lié à une chaîne polyéthylène glycol.

5. Oligonucléotide antisens selon la revendication 1 ou 2, comprenant une base pyrimidine 5-substituée.

6. Oligonucléotide antisens selon la revendication 1 ou 2, comprenant une base 5-méthylcytosine.

7. Composition, comprenant un oligonucléotide antisens selon l'une quelconque des revendications 1-6 et une solution saline qui comprend un tampon phosphate.

8. Oligonucléotide antisens selon l'une quelconque des revendications 1-6, ou composition selon la revendication 7, destiné(e) à être utilisé(e) dans un procédé de traitement de la dystrophie musculaire.

9. Oligonucléotide antisens ou composition destiné(e) à être utilisé(e) selon la revendication 8, où la dystrophie musculaire est la dystrophie musculaire de Duchenne.

FIGURE 1.

bp Acceptor ESE Donor

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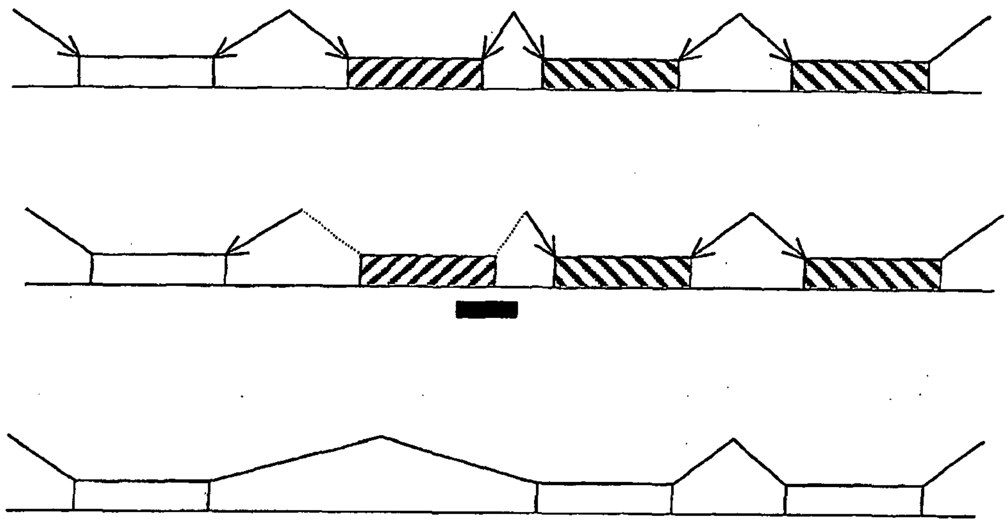
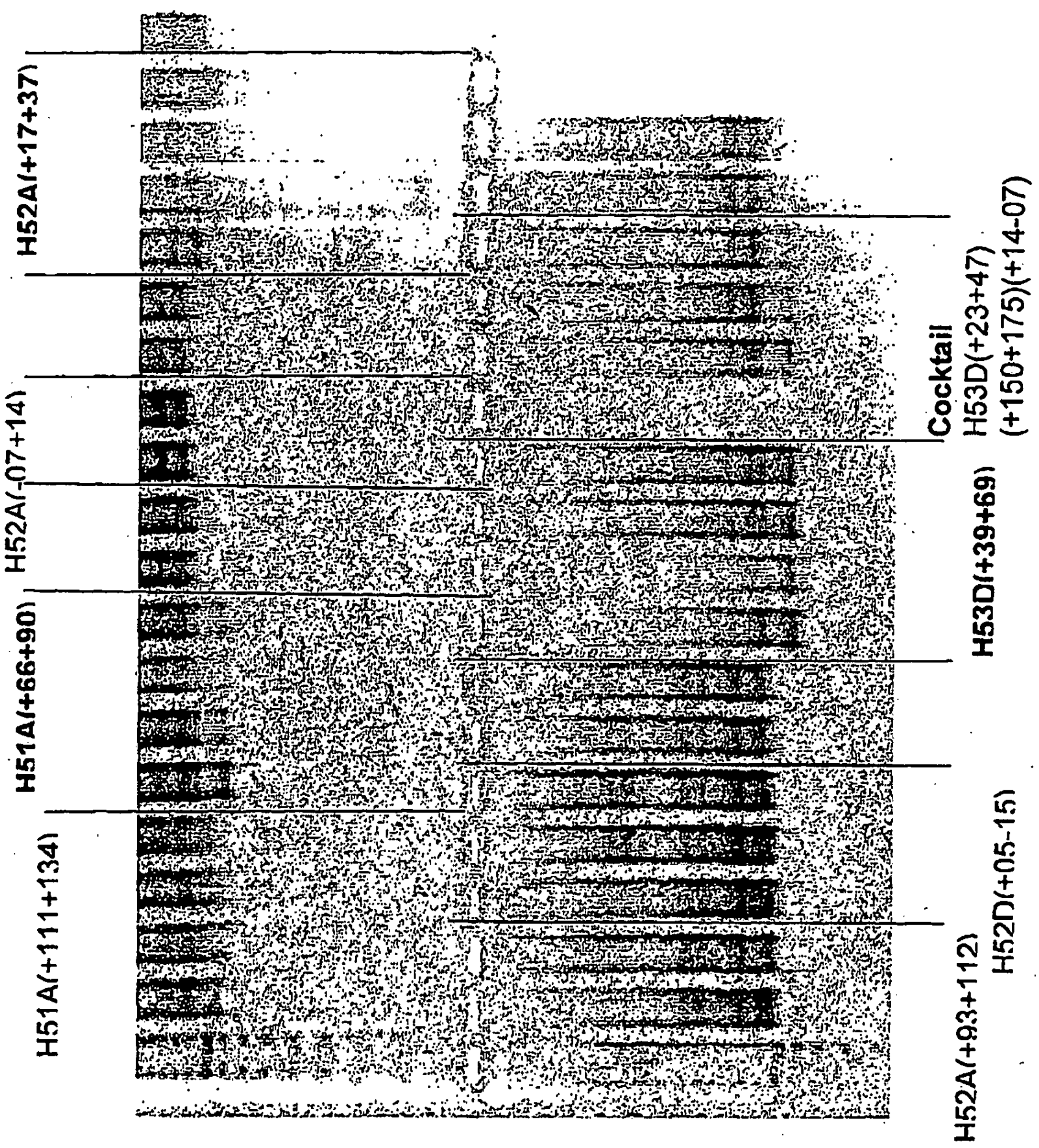


FIGURE 2

FIGURE 3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H52A(-07+14)
H51A(+66+90)
H51A(+111+134)
Cocktail
H53D(+23+47)
(+150+175)(+14-07)
H53D(+39+69)
H52A(+93+112)
H52D(+05-15)

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- CA 2507125 **[0016]**
- WO 2004083446 A **[0016]**
- US 5149797 A **[0059]**
- US 4458066 A **[0066]**
- US 20040248833 A **[0075]**
- US 6806084 B **[0077]**
- EP 10004274 A **[0107]**
- EP 05754344 A **[0107]**
- AU 2005000943 W **[0107]**
- AU 20040903474 **[0107]**

Non-patent literature cited in the description

- **SIERAKOWSKA H et al.** *Proc Natl Acad Sci USA,* 1996, vol. 93, 12840-12844 **[0004]**
- **WILTON SD et al.** *Neuromusc Disorders,* 1999, vol. 9, 330-338 **[0004]**
- **VAN DEUTEKOM JC et al.** *Human Mol Genet,* 2001, vol. 10, 1547-1554 **[0004]**
- **SHERRAT TG et al.** *Am J Hum Genet,* 1993, vol. 53, 1007-1015 **[0006]**
- **LU QL et al.** *Nature Medicine,* 2003, vol. 9, 1009-1014 **[0006]**
- **AARTSMA-RUS A et al.** *Am J Hum Genet,* 2004, vol. 74, 83-92 **[0006]**
- **MATSUO et al.** *J Clin Invest.,* 1991, vol. 87, 2127-2131 **[0009]**
- **TAKESHIMA et al.** *J. Clin. Invest.,* 1995, vol. 95, 515-520 **[0009]**
- **DUNCKLEY et al.** *Nucleosides & Nucleotides,* 1997, vol. 16, 1665-1668 **[0010]**
- **DUNCKLEY et al.** *Human Mol. Genetics,* 1998, vol. 5, 1083-90 **[0011]**
- **ERRINGTON et al.** *J Gen Med,* 2003, vol. 5, 518-527 **[0012]**
- **WILTON et al.** *Neuromuscular Disorders,* 1999, vol. 9, 330-338 **[0014]**
- **MANN CJ et al.** *J Gen Med,* 2002, vol. 4, 644-654 **[0015]**
- **AARTSMA-RUS et al.** *Human Molecular Genetics,* 2003, vol. 12, 907-14 **[0016]**
- **AARTSMA-RUS et al.** *Neuromuscular Disorders,* 2002, vol. 12, 71-7 **[0016]**
- **MANN et al.** *J Gen Med,* 2002, vol. 4, 644-654 **[0034]**
- Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. *J Gen Med,* vol. 4, 644-654 **[0046]**
- **BEAUCAGE et al.** *Tetrahedron Letters,* 1981, vol. 22, 1859-1862 **[0067]**
- **MARTIN.** Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990 **[0071]**
- **MARTIN.** Remington's Pharmaceutical Sciences. Mack Publishing Co, 1990, 1435-1712 **[0072]**
- **MANN CJ et al.** Antisense-induced exon skipping and the synthesis of dystrophin in the mdx mouse. *Proc., Natl. Acad. Science,* 2001, vol. 98 (1), 42-47 **[0076]**
- **GEBSKI et al.** *Human Molecular Genetics,* 2003, vol. 12 (15), 1801-1811 **[0076]**
- **FRALEY et al.** *Trends Biochem. Sci.,* 1981, vol. 6, 77 **[0079]**
- **MANNINO et al.** *Biotechniques,* 1988, vol. 6, 682 **[0080]**
- **FRIEDMANN.** *Science,* 1989, vol. 244, 1275-1280 **[0083]**
- **ROSENBERG.** *Cancer Research,* 1991, vol. 51 (18), 5074S-5079S **[0084]**
- **ROSENFELD et al.** *Cell,* 1991, vol. 68, 143-155 **[0084]**
- **ROSENFELD et al.** *Science,* 1991, vol. 252, 431-434 **[0084]**
- **BRIGHAM et al.** *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0084]**
- **NABEL et al.** *Science,* 1990, vol. 249, 1285-1288 **[0084]**
- **HAZINSKI et al.** *Am. J. Resp. Cell Molec. Biol.,* 1991, vol. 4, 206-209 **[0084]**
- **WANG ; HUANG.** *Proc. Natl. Acad. Sci. (USA),* 1987, vol. 84, 7851-7855 **[0084]**
- **WU ; WU.** *J. Biol. Chem.,* 1988, vol. 263, 14621-14624 **[0084]**
- **WOLFF et al.** *Science,* 1990, vol. 247, 1465-1468 **[0084]**
- *Am. J. Med. Sci.,* 1989, vol. 298, 278-281 **[0084]**
- *Clinical Research,* 1991, 39 **[0084]**
- **ANDERSON.** *Science,* 1992, vol. 256, 808-813 **[0084]**
- **SAMBROOK et al.** Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, 1989 **[0093]**
- DNA Cloning: A Practical Approach. MRL Press, Ltd, 1985 **[0093]**

- **AUSUBEL, F. ; BRENT, R. ; KINGSTON, R.E. ; MOORE, D.D. ; SEIDMAN, J.G. ; SMITH, J.A. ; STRUHL, K.** Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Intersciences, 2002 **[0093]**

EXHIBIT AU

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25. Aug. 2016

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Your reference

Our reference
ESP00349NIP

25 August 2016
K/sh

Re.: European Patent No. 2 206 781 B1
European Patent Application No. 10004274.6
Patentee: The University of Western Australia
Opponent: Nippon Shinyaku Co., Ltd.

On behalf of

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14, Nishinosho-Monguchi-cho, Kisshoin,
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Kyoto 601-8550
Japan

OPPOSITION

is lodged according to Article 99 EPC against the above-referenced patent titled

"Antisense oligonucleotides for inducing exon skipping and methods for use thereof".

The opposition fee amounting to EUR 785,-- is to be debited from our deposit account no. 28000381. A corresponding payment order is enclosed.

The opponent has appointed us as his representatives and it is requested to effect all notifications to our address.

The European patent EP 2 206 781 is opposed in its full extent (claims 1 to 9).

The opposition is based on the grounds of Article 100(a), 100(b) and 100(c) EPC. In particular it is submitted that the patent lacks inventive step. It does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by the skilled person and its subject matter extends beyond the content of the application as originally filed.

It is requested to revoke the European patent in total. Oral proceedings in accordance with Article 116 EPC are requested in the event that the Opposition Division does not reach the decision to revoke the patent on the basis of the written submission of the opponent.

Detailed statement of the grounds for opposition

I. The cited prior art

For substantiation of the opposition it is referred to the prior art documents cited in the opposed patent and during the granting proceedings and the following documents. In particular it is referred to the following documents:

D1 Corey et al., Genome Biology, 2001, 2(5) 1015.1-1015.3

D2 AU 2004903474 (priority document)

D3 WO 2004/083432

D4 WO 2004/048570 (≙ EP 1 568 769)

D5 CA 2 507 125

D6 Aartsma-Rus et al., Human Molecular Genetics (2003), vol. 12, no. 8; pp 907-914

D7 Aartsma-Rus et al., Neuromuscular Disorders vol.12,S71-S77(2002)

D8 experimental report

II. The subject matter of EP 2 206 781

Claim 1 of the opposed patent relates to

a) an isolated antisense oligonucleotide that
   - a_1) binds to human dystrophin pre-mRNA,
   - a_2) wherein said oligonucleotide is 20 to 31 nucleotides in length and

b) is an oligonucleotide that is specifically hybridizable to
   - b_1) an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47),
   - b_2) annealing site H53A (+39+69), or
   - b_3) both

c) wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide and

d) wherein said oligonucleotide induces exon 53 skipping.

Claim 2 relates to an oligonucleotide according to claim 1 which is selected from SEQ ID NOs: 192, 193 and 195, optionally wherein the uracil bases (U) are thymine bases (T).

Claim 3 is referred back to claims 1 or 2 and defines the oligonucleotide further in that it is chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the antisense oligonucleotide.

Claim 4 is referred back to claim 3 and defines the moieties or conjugates wherein the oligonucleotide is chemically linked to a polyethylene glycol chain.

Claim 5 is referred back to claim 1 or 2 and mentions that it comprises a 5-substituted pyrimidine base.

Claim 6 is referred back to claim 1 or 2 and mentions that it comprises a 5-methylcytosine base.

Claim 7 relates to a composition comprising an oligonucleotide as defined in any of claims 1-6 and a saline solution that includes a phosphate buffer.

Claim 8 relates to an antisense oligonucleotide according to any of claims 1-6, or a composition of claim 7, for use in a method of treatment of muscular dystrophy.

Claim 9 is referred back to claim 8 and defines the muscular dystrophy further in that it is Duchenne Muscular Dystrophy.

It is alleged that the opposed patent provides a target region within exon 53 spanning the annealing sites H53A (+23+47) and/or H53A (+39+69) with a morpholino antisense oligonucleotide ("PMO") of 20 to 31 nucleotides in length. This target region is efficient in vivo splicing (compare with patentee's submission dated September 17, 2014). It is alleged that the key region of exon 53 can be targeted to provide therapeutically effective exon skipping.

III. Article 100(c)/123(2) EPC / added matter

3.1 Intermediate generalization

The granted claims have to be revoked in view of an inadmissible intermediate generalization since by the amendments features were taken out of the initial context and combined with others. Such "intermediate generalization" is considered to be inadmissible (see e.g. T962/98, T1408/04, T461/05 or T1118/10 to name only a few of the relevant decisions).

3.1.1 Three annealing sites

Claim 1 contains three annealing sites, namely

1) H53A (+23+47) which corresponds to feature b_1) and
2) annealing site H53A (+39+69) as annealing site b_2) and furthermore
3) both annealing sites (feature b_3)).

First of all it should be mentioned that feature b_3), namely both annealing sites, is obviously nowhere disclosed. To be more precise, the word "both" in combination with the two annealing sites representing features b_1) and b_2) of claim 1 can nowhere be found in the specification as originally filed (WO 2006/000057).

For the interpretation what the term "both" should mean according to the interpretation of patentee we refer to the submission of patentee's representatives dated September 17, 2014, page 2, first paragraph. This paragraph reads as follows:

"*The solution provided by the present invention is to target a region within exon 53 spanning the annealing sites H53A (+23+47) and/or H53A (+39+69) with a morpholino antisense oligonucleotide ('PMO') of 20-31 nucleotides in length.*"

From this statement it can be concluded that it is the intention of patentee to claim not only one of the two annealing sites (feature b_1) or b_2), respectively), but also the area from nucleotide +23 until +69. This is supported by Figure A which was also submitted on November 17, 2014 by patentee's representatives. This "area" is nowhere disclosed in the application as originally filed. Therefore, Article 123(2) EPC is violated.

According to feature a_2) the claimed oligonucleotide should be 20-31 nucleotides in length. Concerning the length of the oligonucleotides a paragraph on page 21, lines 9-17 of WO 2006/000057 can be found. This passage generally says that the antisense oligonucleotides can be as short as 12 bases whereas such length are not as efficient as longer (20-31 bases) oligonucleotides. The selection of 20-31 bases is therefore a selection of a list of various lengths of oligonucleotides. When we consider the relevant passage on page 62 of WO 2006/000057 there is no range of oligonucleotides provided. Table 39 summarizes 12 antisense oligonucleotides having different lengths. It should be noted that only two target regions are features of the claims, namely b_1) and b_2). The antisense oligonucleotide H53A (+23+47) has a length of 25 bases and the oligonucleotide H53A (+39+69) has a length of 31 bases. Those two embodiments corresponding to SEQ ID NO: 193 and 195 cannot form a basis for the range of 20-31 nucleotides.

It seems that the feature that the antisense oligonucleotide is a morpholino antisense oligonucleotide (feature c) of claim 1) has a potential basis on page 17, line 3 of the published international application. This passage relates, however, to Table 1A wherein all sequences designated as SEQ ID NOs: 1-211 are summarized. Alternatively in the relevant sentence on page 17, lines 2-4, other antisense chemistries such as peptide nucleic acids are, however, mentioned.

A basis for feature d), namely that the oligonucleotide induces exon 53 skipping might potentially be found on page 62 and in particular Table 39 of WO 2006/000057.

In Table 39 there are mentioned several antisense oligonucleotides whereby nine oligonucleotides are designated as H53A. The splice sites at the beginning and end of the exon are, however, different.

Patentee has selected from Table 39, which can be considered as a list, among nine individual oligonucleotides only two and the reason for selecting features b_1) and b_2), respectively, is not evident. When we look at the biological activity (ability to induce skipping) H53A (+39+69) corresponding to SEQ ID NO: 193 (feature b_2)) is said to induce strong skipping to 50 nM. The annealing site of feature b_1), namely H53A (+23+47) corresponding to SEQ ID NO: 195 is said to induce only very faint skipping to 50 nM.

According to claim 2 SEQ ID NO: 192 which corresponds to H53A (+36+62) should also fall under claim 1 and shows only a faint skipping at 50 nM (compare with Table 39).

Considering the other biological activities, namely the ability to induce skipping of other antisense oligonucleotides we noticed that for example H53A (+45+69) can induce faint skipping at 50 nM or H53A (+150+176) can also induce very faint skipping at 50 nM.

Therefore, features b_1) and b_2), respectively, were arbitrarily selected and it seems that the selected oligonucleotides do not fulfill the feature d) of claim 1, namely that the oligonucleotide induces exon 53 skipping since Table 39 shows varying degrees of biological activity down to very faint skipping.

3.1.2 Specifically hybridizable

When considering the term "specifically hybridizable to" in claim 1 it is more likely that also oligonucleotides that have little or faint skipping activities were used as basis. The description only provides a vague definition for "specifically hybridizable" in paragraphs [0052] and [0053], reading, e.g. *"a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target"* (see para. [0052]).

The specification as originally filed defines on page 23, lines 16-31 the term "specifically hybridizable". An antisense molecule is considered to be specifically hybridizable when binding of this compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility and there is a specific degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e. under physiological conditions in the case of in vivo assays or in the case of in vitro assays.

An oligonucleotide is therefore only "specifically hybridizable" when two conditions are fulfilled:

1) The antisense molecule must specifically hybridize with the target DNA or RNA and

2) Non-specific binding of the antisense compound to non-target sequences must be avoided. It is evident that this second condition requires that it had to be tested whether an oligonucleotide does bind or does not bind in a non-specific way to non-target sequences.

As potential basis for claim 1 pages 62-63 of the originally filed documents may serve. This section can, however, not serve as a basis for the feature "specifically hybridizing" for three reasons:

1) The strength of binding by hybridization and skipping is not the same;

2) Only the antisense oligonucleotide designated as H53A(+39+69) is said to induce strong skipping whereas the other oligonucleotides show only faint or very faint skipping speaking for a comparatively weak hybridization;

3) There is no disclosure whatsoever that those oligonucleotides do not specifically bind to non-target sequences.

Therefore, the passage in the general part of the specification on page 23 cannot be combined with the disclosure of Table 39. With other words: There is no disclosure that the antisense oligonucleotides disclosed on page 62 have the feature of "specifically hybridize to".

3.2 <u>Selection from at least two lists</u>

The opposed patent is to be revoked since granted claim 1 is an inadmissible selection from more than two lists of features which violates the requirements of Article 123(2) EPC (e.g. T223/11, T1651/11).

The combination of features created by patentee is not admissible under established jurisprudence of the Boards of Appeal considering the aspect that the features are selected from several lists. This is not allowable in view of the quoted decisions of the Board of Appeal. Of course there are many more T-decisions which could also be quoted in order to support this argument.

Furthermore, dependent sub-claims 2-9 are directly or indirectly referred back to claim 1. There is no disclosure wherein the features are originally disclosed in combination. Therefore, patentee has selected the features from different lists (e.g. former subclaims) which is a clear violation of the established case law of the Boards of Appeal.

The objected claims have therefore to be revoked, because the presently claimed combination of this feature is not disclosed and the claims are considered to be an artificially created new embodiment (e.g. T 2496/10, point 4.6).

IV. Article 100(b) EPC

Claim 1 relates to oligonucleotides with 20 to 31 nucleotides in length. It is submitted that the claimed invention is not enabled over the whole scope of the claim, in particular for oligonucleotides having 20 to 24 oligonucleotides.

The whole scope of the claim is not supported by the description, in view of the low predictability in the art, especially for the annealing range of +39+69, because the description provides data for only 191(+45+69),192(+39+62) and 193(+39+69) but no others.

These relevant oligomers from Table 39 have the length of 25, 24 and 31 base pairs respectively. In view of this, the description of the opposed patent fails to support whether oligonucleotides having the length of less than 24 base pairs actually have skipping activity. In this context, Corey and Abrams (Genome Biology 2001, 2 (5); 1015.1-1015.3, submitted as document **D1**) teach for morpholino antisense oligonucleotides that "this binding is no tighter than binding of analogous DNA and RNA oligomers, necessitating the use of relatively long 25-base morpholinos for antisense gene inhibition" (page 1015.1, left column, 4th to 2nd lines from the bottom).

In the absence of experimental data for oligonucleotides having the length of less than 24 base pairs, and in view of the teaching by Corey and Abrams (**D1**), it must be concluded that oligonucleotides having the length of less than 24 base pairs are not supported in the description.

Furthermore, it should be stressed that the only oligonucleotide which shows according to Table 39 a strong skipping ability has a length of 31 nucleotides. Contrary thereto shorter oligonucleotides like H53D(+14-07) having a length of 21 oligonucleotides or H53A(-12+10) having a length of 22 oligonucleotides or H53A(+07+26) having a length of 20 oligonucleotides

show only a very faint skipping or no skipping ability at all. Therefore, the opposed patent is to be revoked in view of lack of enabling disclosure.

V. Priority

The opposed patent claims the priority of the Australian patent application no. 2004903474 as filed on June 28, 2004. None of the claims is entitled to the priority of the Australian patent application. Claim 1 relates to two annealing sites (feature b_1) and feature b_2)) or both (feature b_3)). None of those annealing sites has a basis in the priority document.

The oligomers disclosed in the priority application are summarized in Table 1 in the specification of the priority document submitted as **D1**. Table 1 is shown in the following:

Brief Description of the Sequence listings

SEQ ID	SEQUENCE	NUCLEOTIDE SEQUENCE (5' - 3')
1	H8A(-06+18)	GAU AGG UGG UAU CAA CAU CUG UAA
2	H8A (-03+18)	GAU AGG UGG UAU CAA CAU CUG
3	H8A(-07+18)	GAU AGG UGG UAU CAA CAU CUG UAA G
4	H8A(-06+14)	GGU GGU AUC AAC AUC UGU AA
5	H8A(-10+10)	GUA UCA ACA UCU GUA AGC AC
6	H7A(+45+67)	UGC AUG UUC CAG UCG UUG UGU GG
7	H7A(+02+26)	CAC UAU UCC AGU CAA AUA GGU CUG G
8	H7D(+15-10)	AUU UAC CAA CCU UCA GGA UCG AGU A
9	H7A(-18+03)	GGC CUA AAA CAC AUA CAC AUA
10	C6A(-10+10)	CAU UUU UGA CCU ACA UGU GG
11	C6A(-14+06)	UUU GAC CUA CAU GUG GAA AG
12	C6A(-14+12)	UAC AUU UUU GAC CUA CAU GUG GAA AG

13	C6A(-13+09)	AUU UUU GAC CUA CAU GGG AAA G
14	CH6A(+69+91)	UAC GAG UUG AUU GUC GGA CCC AG
15	C6D(+12-13)	GUG GUC UCC UUA CCU AUG ACU GUG G
16	C6D(+06-11)	GGU CUC CUU ACC UAU GA
17	H6D(+04-21)	UGU CUC AGU AAU CUU CUU ACC UAU
18	H6D(+18-04)	UCU UAC CUA UGA CUA UGG AUG AGA
19	H4A(+13+32)	GCA UGA ACU CUU GUG GAU CC
20	H4D(+04-16)	CCA GGG UAC UAC UUA CAU UA
21	H4D(-24-44)	AUC GUG UGU CAC AGC AUC CAG

Table 1: Description of 2'-O-methyl phosphorothioate antisense oligonucleotides that have been used to date to study induced exon skipping during the processing of the dystrophin pre-mRNA.

Only skipping of exons 8, 7, 6 and 4 is attempted in the priority application.

Thus, exon 53 skipping and oligomers therefor, H53A (+23+47) and H53A (+39+69), are not disclosed in the priority application and the claims are therefore not entitled to the priority of June 28, 2005.

As the H53A (+23+47) and H53A (+39+69), as exemplary embodiments of claim 1, are not entitled to the priority, inventiveness of claims 1 to 9 is to be evaluated as of the international filing date of **28 June 2005**.

VI. Inventive Step

6.1 WO 2004/083432 (**D3**)

Claim 1 of the opposed patent relates to

a) an isolated antisense oligonucleotide that

   a_1) binds to human dystrophin pre-mRNA,

   a_2) wherein said oligonucleotide is 20 to 31 nucleotides in length and

b) is an oligonucleotide that is specifically hybridizable to

   b_1) an exon 53 target region of the Dystrophin gene designated as annealing site H53A (+23+47),

b_2) annealing site H53A (+39+69), or

b_3) both

c) wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide and

d) wherein said oligonucleotide induces exon 53 skipping.

D3 (WO 2004/083432) discloses an antisense oligomer named "h53AON1", which enables exon 53 skipping and has the following sequence (see Table 2 on page 48):

Table 2
Characteristics of the AONs used to study the targeted skipping of 15 different DMD exons*

Name	Antisense sequence (5'-3')	Length (bp)	G/C%	U/C%	Exon skip	Transcript
h53AON 1	cuguugccuccgguucug	18	61	72	+	OF

The complementary sequence of h53AON1 is CAGAACCGGAGGCAACAG. This sequence is completely encompassed in the claimed annealing sites b_2) and b_3) of the subject patent (see below where the yellow highlighted part of b_2) corresponds to the complementary sequence of h53AON1).

b_2): **CACCTTCAGAACCGGAGGCAACAGTTGAATG**

Therefore, **D3** discloses antisense oligonucleotide having feature b).

In addition, h53AON1 is shown to have a high skipping activity (see Figure 1E etc.). **D3** also discloses that the oligomers can be modified with morpholino phosphorodiamidate (see page 10, line 11 to page 11, line 4). Thus, the features c) and d), i.e. "wherein said antisense oligonucleotide is a morpholino antisense oligonucleotide, and, wherein said oligonucleotide induces exon 53 skipping", are disclosed in **D3**.

h53AON1 per se has a length of 18 oligonucleotides and does not satisfy requirement a_2) "wherein said oligonucleotide is 20 to 31 nucleotides in length".

However, **D3** states "*[c]urrently, many different compounds are available that mimic hybridization characteristics of oligonucleotides. Such a compound is also suitable for the present invention is such equivalent comprises similar hybridization characteristics in kind not necessarily in amount….As mentioned, oligonucleotides of the invention do not have to consist of only oligonucleotides that contributes to hybridization to the targeted exon. There may be additional material and/or nucleotides added.*" (see page 12, lines 11 to 20 of **D3**).

Based on the above statement, an average person of the skill in the art would have easily conceived of a "modification" of h53AON1 having additional nucleotides added, which may well be of the length of 20 to 31 nucleotides. Further, when two nucleotides are added to h53AON1 to make a 20mer equivalent, with any combination of those two nucleotides, at least 90% of the entire stretch of the 20mer equivalent is still completely identical to the counterpart region of the b_2) annealing site. Thus, it is clear that such 20mer is "specifically hybridizable to" b_2).

Considering the teaching by Corey and Abrams (**D1**) wherein a longer oligonucleotide is recommended for skipping (see supra), an average person skill in the art would have been motivated to modify h53AON1 by adding extra nucleotides to make it longer.

Therefore, the subject matter of claim 1 lacks inventive step over **D3** since **D3** contains the suggestion to elongate short nucleotides.

5.2 <u>WO 2004/048570 (**D4**) in view of **D3**</u>

The international patent application WO 2004/048570 was originally published in Japanese language on September 30, 2004. The document is therefore pre-published prior art. The English translation of this document was published as EP 1 568 769 and we refer in the following to this English document as **D4**.

D4 discloses oligomers enabling exon 53 skipping (see Examples 79-87), among which AO95 of Example 87 having SEQ ID NO: 75: corresponds to the 30th to 47th nucleotides of exon 53.

SEQD ID NO:75 of the sequence listing shows the nucleotide sequence of the oligonucleotide prepared in Example 87 (A095) (page 216, line 29).

<210> 75
<211> 18
<212> DNA
<213> synthetic oligonucleotide

<400> 75
ctgaaggtgt tcttgtac

The complementary sequence of AO95 is GTACAAGAACACCTTCAG.

This sequence is completely encompassed in the claimed annealing sites b_1) and b_3) of the subject patent (see below where the yellow highlighted part of b_1) corresponds to the complementary sequence of AO95).

b_1): **GGATGAAGTACAAGAACACCTTCAG**

Thus, AO95 is contained within SEQ195 (+23+47) of the subject claim. AO95 is demonstrated to have a skipping activity (see Figure 19 and para. [0319] of EP1568769A1). **D4** also discloses that the compound may be of morpholine salt (see para. [0046] of EP1568769A1).

AO95 per se is in the length of 18mer and does not comply with requirement a_2) "wherein said oligonucleotide is 20 to 31 nucleotides in length". However, **D3** states "*[c]urrently, many different compounds are available that mimic hybridization characteristics of oligonucleotides. Such a compound is also suitable for the present invention is such equivalent comprises similar hybridization characteristics in kind not necessarily in amount....As mentioned, oligonucleotides of the invention do not have to consist of only oligonucleotides that contributes to hybridization to the targeted exon. There may be additional material and/or nucleotides added.*" (see page 12, lines 11 to 20 of **D3**).

Based on the above statements of **D3**, a person skilled in the art would have easily conceived of a variant of AO95 having additional nucleotides added, which may well be of the length of 20-31. Further, when two nucleotides are added to AO95 to make a 20mer equivalent, with any combination of those two nucleotides, at least 90% of the entire stretch of the 20mer equivalent is still completely identical to the counterpart region of the b_1) annealing site. Thus, it is clear that the 20mer equivalent "specifically hybridizable to" b_1).

A person skilled in the art starting from the specific disclosure as explained above in document **D3** or **D4**, respectively, and looking for an alternative embodiment has a strong motivation to elongate the 18mers as disclosed in **D3** or **D4**, respectively, by at least 2-10 oligonucleotides since it can be expected that the binding of the oligonucleotide will be improved by such an elongation as taught by **D3** or **D1**, respectively.

Therefore, the claimed invention is rendered obvious for the average person skilled in the art by either a combination of **D3** and **D4,** or a combination of **D3** and **D1**.

5.3 <u>The subject matter of claim 1 does not exert superior effect in full scope.</u>

"h53AON1" has been cited as a prior art by the Examining Division of EPO in the examination stage of the subject patent. In the opinion attached to the extended European search report dated January 2, 2013 the searching authority cited inter alia documents D1, D2 and D3. These documents are submitted as document **D5** (CA 2 507 125), **D6** (Human Molecular Genetics, vol. 12 (2003), pages 907-914) and **D7** (Neuromuscular Disorders, vol. 12, January 2002, pages S71-S77). In section 3 the searching authority concluded that all of documents D1-D3 (now corresponding to **D5-D7**) disclose antisense oligonucleotides targeting exon 53 in the region corresponding to H53A (+23+47) and H53A (+39+69) for inducing exon skipping in the dystrophin gene in order to treat DMD.

Regarding "h53AON1", the University of Western Australia argued that H53A (+39+69) is superior over h53AON1 in terms of skipping activity (see Annex 2 - response filed by the representatives of the University of Western Australia dated 5 November 2013).

Nippon Shinyaku (NS) conducted experiments to see whether any oligomers falling within the scope of claim 1 of the subject patent have superior activity over h53AON1 and found that some oligomers have inferior activities to h53AON1. The experimental report was performed by opponent and is submitted as document **D 8**.

The data as shown in the experimental report submitted as **D8**, confirm that the alleged superior activity as argued by the patentee in the examination stage is not obtainable over the whole scope of the claim, and thus the claimed subject matter is not inventive.

Since no superior effect has been shown over the closest prior art the object of the opposed patent is to provide an alternative oligonucleotide. Such an alternative is, however, rendered obvious by the prior art, in particular by a combination of **D3** and **D1** or **D4** and **D3**.

The claimed subject matter is not inventive in view of the relevant prior art.

Conclusion

Since the opposed patent violates the European patent convention in several respect, the complete revocation of the opposed patent is justified.

Dr. G. Keller

Enc.:
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Your reference	Our reference	
	ESP00349NIP	29 September 2017 K/sh

Re.: European Patent No. 2 206 781 B1
European Patent Application No. 10004274.6
Patentee: The University of Western Australia
Opponent: Nippon Shinyaku Co., Ltd.

This is in response to the summons to attend oral proceedings pursuant to Rule 115(1) EPC dated March 30, 2017.

I. Main Request

1.1 <u>Article 123(2) EPC</u>

The Opposition Division expressed its preliminary opinion that the Main Request extends beyond the disclosure of the application as originally filed (in respect of the binding of both sites, the morpholinos and the combination of morpholinos with any given oligonucleotide targeting the two sites H53A(+23+47) and H53A(+39+69)). Opponent agrees with this preliminary opinion. In the following we would like to comment the arguments of patentee submitted on February 22, 2017:

1.1.1 Intermediate generalization

Claim 1 comprises several features of the isolated antisense oligonucleotide which do either not have any literal support or are picked from several different passages of the original specification whereby it is not disclosed that all features relate to a preferred embodiment.

1.1.1.1 Three annealing sites

Claim 1 comprises three annealing sites whereby feature b_3 relates to "both" annealing sites, namely H53A(+23+47) and H53A(+39+69). Patentee was not able to refer to a literal disclosure but argued that it is allegedly not required to have a strict literal basis for the term "both" provided such feature can be directly and unambiguously derived from the content of the application as filed.

We strongly disagree with this argument since none of the passages quoted by the patentee may serve as a direct and unambiguous support for the feature "both".

a) Table 39

Patentee refers to Table 39 on pages 62 and 63 of the specification as filed and alleges that a person skilled in the art would, on viewing the application and the data as a whole, directly and unambiguously understand that the target annealing site encompasses both, namely H53A(+23+47) and H53A(+39+69). Patentee does, however, not explain the reason <u>why</u> the person skilled in the art should understand that <u>both</u> annealing sites should be suitable. In Table 39 there is only one AON which is disclosed to have a strong skipping activity, namely H53A(+39+69). Furthermore, there are two AONs, namely H53A(+45+69) and H53A(+39+62) which are disclosed to have a faint skipping activity. Those two AONs are located in the same annealing site (HA53A(+39+69)).

Furthermore, there are three AONs disclosed which are said to have very faint skipping activity, namely H53A(+23+47) which corresponds to the first annealing site and two AONs which are outside of both annealing sites, namely H53D(+14-07) and H53A(+150+176). From the "very faint skipping" ability the person skilled in the art may speculate that there are at least three other regions which may potentially be interesting. The person skilled in the art learns on the other hand from Table 39 that variations in the oligonucleotide positions of the AONs may easily lead to a complete loss of skipping activity since several (4) of the tested AONs did not show any skipping activity at all.

Patentee's argument that four out of seven AONs in Table 39 skipped exon 53 is not convincing since there are also three other AONs which showed a faint skipping and which are located outside of the claimed annealing site. Since the ability to induce skipping varies substantially from strong skipping to very faint skipping and since very faint skipping is reported to occur also at other annealing sites, e.g. H53D(+14-07) and H53A(+150+176), the

person skilled in the art cannot deduce unambiguously and directly from this Table that both annealing sites of claim 1 are suitable to perform exon skipping. To the contrary it is not possible to conclude from the data of Table 39 directly and unambiguously that there is a binding region which may be directly and unambiguously derived from the AONs of Table 39.

b) Page 36, lines 19-20

Patentee referred to page 36, lines 19-20 of the application as filed wherein is said that once efficient exon skipping had been induced with one antisense molecule, subsequent overlapping antisense molecules may be synthesized and then evaluated in the assay as described above.

This sentence may be understood as an invitation to perform a research programme and to synthesize and study several different AONs and to check whether skipping occurs or not. This is, however, not a clear and unambiguous disclosure of a combination of both annealing sites as claimed in claim 1 of the Main Request. It should also be mentioned that cited T 0667/08 does not support patentee's position. This decision states in section 4.1.4:

"*... it is therefore essential, when deciding on issues of added subject-matter, to identify the actual teaching conveyed by the original disclosure, i.e. the technical information that the skilled person reading the original disclosure would have derived from its content (description, claims, drawings) considered in its entirety.*

This approach might lead to the identification of subject-matter which has not been explicitly revealed as such in the application as filed, but nevertheless derives directly and unambiguously from its content. Literal support is not required by the wording of Article 123(2) EPC. An amendment can therefore be allowable if it combines information which has not been disclosed in one and the same section of the original disclosure, but results, for instance, from information gathered from various embodiments possibly associated with general statements regarding the information derivable from the introductory section of the application."

The information which is objectively derivable from Table 39 does not lead the person skilled in the art directly and unambiguously to the feature b_3 (both) of claim 1. Therefore, we believe that the Opposition Division is perfectly correct in its preliminary opinion that the term "both" is not directly and unambiguously derivable from the content of the application as originally filed.

1.1.1.2 Nucleotides in length

As basis for the length of the nucleotides patentee referred to page 21, second paragraph of WO 2006/000057. It seems that this is the only passage where different lengths of the antisense oligonucleotides are disclosed. The sentence starting on page 21, line 12 refers to targets such as exon 19 and it continues to disclose that short oligonucleotides such as 12 bases were able to induce exon skipping which were, however, not as effective as longer oligonucleotides (20-31 bases). In the second sentence it is disclosed that in some other targets antisense oligonucleotides having 23, only 17 or 25 nucleotides were disclosed. The quoted sentences teach the person skilled in the art that for the different exons various lengths of the antisense oligonucleotides were used. Interestingly, however, in this passage exon 53 is not even mentioned. Therefore, patentee has selected from a list of different lengths of oligonucleotides, namely 12 bases, 20-31 bases, 17 oligonucleotides and 25 oligonucleotides one range and combined it with an exon which is not even mentioned in this paragraph. Patentee has therefore selected from a list of about 50 exons one exon and has combined this with one length of oligonucleotides out of four different options although the selected length of the oligonucleotides was disclosed in connection with exon 19 which is not subject of claim 1.

It is more than evident that patentee has selected features from two passages (lists) of the original disclosure and combined them although originally no relationship between the two features was disclosed.

1.1.1.3 Morpholino

It is true that on page 17, line 3 morpholinos are mentioned. The quoted sentence makes, however, clear that for certain antisense chemistries such as peptide nucleic acids or morpholinos the uracil (U) bases may be shown as thymidine (T).

It should, however, be kept in mind that the opposed patent describes very detailed the modification of the oligonucleotides. In order to avoid degradation of pre-mRNA during duplex formation with the antisense molecules the antisense molecules used in the method of the opposed patent may be adapted to minimize or prevent cleavage by endogenous RNase H (page 25, lines 15-17 of the application as originally filed).

There are many different modifications disclosed in the passage starting on page 25, line 15 and ending on page 28, line 6 of WO 2006/000057. To select only the morpholinos and to combine this feature with the target regions (features b) of claim 1) is a further selection from a further different list.

1.1.1.4 Inducing exon skipping

Patentee referred to Table 39 on pages 62-63 of the application as originally filed. It should be noted, however, that this Table does not directly point to a binding region of exon 53 since there are several antisense oligonucleotides disclosed which are directed to exon 53 but do not induce skipping, namely H53A(-12+10), H53A(-07+18), H53A(+07+26) and H53A(+124+145). Those results have simply not been mentioned by patentee.

It should be kept in mind that the patentee has made an inadmissible selection from at least four lists, namely

a) the exon,
b) the modification of the oligonucleotide,
c) the length of the oligonucleotides in claim 1, and
d) the annealing sites.

1.1.1.5 Specifically hybridizable

The relevant passage wherein the term "specifically hybridizable" is disclosed in the application as originally filed (page 23, lines 16-31) has been cited correctly by patentee on page 4 of the last submission. Our argument with regard to Article 123(2) EPC is that this passage is according to the original disclosure related to each and every oligonucleotide as disclosed in the application of the opposed patent. It is, however, not related to oligonucleotides which bind to a particular annealing site in exon 53 and which have a specific length.

The inadmissible broadening is more evident when this passage is read in combination with the disclosure of Table 39. The quoted passage on page 23, lines 16-31 of the original disclosure broadens the claim by allowing oligonucleotide substitutions so that the oligonucleotide must not be 100% complementary to the target sequence whereby only unspecific binding should be avoided.

To combine this passage with the disclosure of Table 39 may have the consequence that the sequence of the provided antisense oligonucleotides may be changed by nucleotide substitutions. The person skilled in the art will, however, learn from Table 39 wherein only one oligonucleotide shows a strong skipping ability and all other oligonucleotides show only a faint, very faint or no skipping ability that it is very likely that the skipping ability will be dramatically reduced if changes of the oligonucleotide sequence are performed. There is no reason for the skilled artisan to believe that modifications of the AONs might have the ability to include skipping.

1.1.1.6 Selection from two lists

Patentee referred to T 783/09 in order to argue that the features of opposed claim 1 are not selected from two lists in an inadmissible manner. In T 783/09 the Board decided that a claim comprising only three out of 44 combinations derivable from two lists was allowable. The factual situation of T 783/09 is, however, completely different from the present case since a selection was made from several different lists such as number of exon, length of oligonucleotides, target site, selectively hybridizable and modification of the oligonucleotide in order to obtain the combination of features of claim 1. The number of combinations derivable from the lists amounts to several thousands of potential combinations. Therefore, T 783/09 is not applicable for the present case.

1.1.2 Dependent claims

It is true that we did up to now not analyze in detail the features of claims 2 to 9. The reason therefore is that all claims are directly or indirectly dependent from claim 1. If claim 1 has to be revoked due to violation of Article 123(2) EPC the dependent claims will share this fate. In case the objections of claim 1 can be overcome we reserve the right to present the then relevant arguments with regard to the features of the claims dependent from claim 1.

As an intermediate result it can be stated that claim 1 has to be revoked due to a violation of Article 123(2) EPC for several reasons and the claims dependent from claim 1 share this fate.

1.2 <u>Article 100(b)/83 EPC</u>

In its reply (page 8) patentee has argued that the burden of proof for non-enablement lies with the opponent and that the opponent has filed no data to support the allegation that AONs of 20 to 24 nucleotides would not work. We do not agree with patentee since we have presented serious doubts, substantiated by verifiable facts.

1.2.1 Evidence which causes serious doubts

1.2.1.1 (D1)

We have already submitted document D1 which has been published before the priority date of the opposed patent. It is true that the morpholino antisense oligonucleotides are described as tools for investigating vertebrate development. On the other hand the molecular mechanisms are based on molecular principles which are in general applicable in vertebrate organisms. Therefore, the statement of D1 that relatively long oligomers (morpholinos) of at least 25 nucleotides are required for exon skipping is valid.

1.2.1.2 (D9)

Document D9 was cited by patentee and published after the filing date of the opposed patent confirms the finding of D1. On page 108, right column, lines 1-9 from the bottom, the following sentence can be found:

"*Indeed, the fact that the 30 mer PMOs (-G,-H) were more bioactive than the 25 mer PMO counterpart (-A) targeted to the same open/accessible sites of the exon would suggest that strength of binding of PMO to the target site may be the most important factor in determining PMO bioactivity.* ***<u>The influence of AO length on bioactivity has been reported elsewhere [9,30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart.</u>***" (emphasis added)

The article continues on page 109, left column:

"*The reason that our PMOs produce higher levels of exon skipping could be a (combined) consequence of the different AO chemistries, length of AO used, type of cell used (patient vs. control) and the absolute target site of AO.*"

Consequently the literature teaches the person skilled in the art that short oligonucleotides of 20 to 23 oligonucleotides will have no skipping activity. The opposed patent does not contain an example showing the contrary.

From the data provided in Table 1 and Figure 1 it can be learned that the 30 mer PMO-G and PMO-H produce higher levels of skipping relative to the 25 mer PMO-A and PMO-B. Consequently a further reduction of skipping activity can be expected when the AOs are further shortened.

1.2.1.3 (D8)

Opponent has also provided evidence (D8) showing that short antisense oligomers having a length of 18 to 21 nucleotides do not induce sufficiently exon 53 skipping. Therefore, there are serious doubts which have been substantiated by verifiable facts that the claimed invention cannot be worked over the whole claimed range, in particular when short AONs are used.

1.2.2 "Specifically hybridizable"

Claim 1 is substantially broadened by the feature "specifically hybridizable to an exon 53 target region" which means that the claimed antisense oligonucleotide must not match exactly to the exon 53 target region, but also such AONs are encompassed which must only have a sufficient degree of complementarity that a stable and specific binding occurs between the oligonucleotide and the DNA or RNA target.

By using the feature "specifically hybridizable" the claimed oligonucleotide must not be 100% complementary to the target sequence ([0053], first sentence). Therefore, at least one or even more nucleotides can be replaced by other nucleotides which do not perfectly match with the target sequence. The consequence of lowering the complementarity to a value below 100% is necessarily that the skipping ability will be reduced. When very faint skipping ability is further reduced, because the complementarity of antisense molecule and target sequence is below 100% the skipping ability will not be only faint but also hardly be detectable.

Patentee has not provided any evidence that an oligonucleotide which is not 100% complementary to the target sequence shows any skipping activity. In view of the serious doubts which are substantiated above the burden of proof that the requirements of Art. 83 EPC are met rests on patentee.

Since opponent has provided evidence which causes serious doubts substantiated by verifiable facts that the claimed invention cannot be worked over the whole claimed range, the burden of proof rests with patentee to produce sufficient evidence that the claimed invention is disclosed in an enabling manner over the whole scope of the claims, in particular with regard to comparatively short oligonucleotides of 20 mer to 25 nucleotides which are not 100% complementary to the target sequence.

1.3 Article 56 EPC (inventive step)

The arguments of patentee relating to inventive step can be found in sections 3.0 (Background to the Invention, pages 6, 7) and 6.0 (Inventive Step, pages 9-14).

1.3.1 Post-published evidence (D9)

It seems that patentee tries to transfer disclosure from the post-published document D9 into the disclosure of the present patent in order to bolster the argumentation relating to inventive step. In general the disclosure of post-published documents can only be taken into account for the question of sufficiency of disclosure if it was used to back up the positive findings in relation to the disclosure of a patent application (T 1273/09 citing T 609/02).

It is, however, not admissible to combine the teaching of an application with the teaching of post-published evidence in order to support the presence of an inventive step or to transfer a statement from post-published evidence (D9) into the disclosure of the opposed patent as suggested on page 7 of applicant's submission dated February 22, 2017.

Contrary to patentee's allegation the opposed patent fails to state that targeting the claimed sequence between nucleotides 23 and 69 or between 30 and 65, respectively, results in skipping of exon 53.

Two arguments strongly contradict patentee's assertion:

First, the experimental section of the present specification reports that some antisense oligonucleotides have only a faint or very faint ability to induce skipping.

Second, D9 notes that the levels of exon skipping that may be considered effective is over 50% exon skipping (compare with page 108, right column, lines 14-17). Consequently D9 also

discloses that among 13 PMOs tested, only 6 PMOs, e.g. G, H, A, I, B and M show activities over 50% whereas other PMOs show lower activities. For example D9 describes that PMO-J has the activity of only 37% and that all other PMOs tested gave exon skipping at levels of between 15% and 26% (see page 104, right column, lines 12-14). That means that 6 out of 13 PMOs tested which is more than half of those tested show activities less than 50%, more precisely less than 40%. In addition D9 reports that even the PMOs with high skipping activity at one concentration also show the skipping activity as low as 30% at lower concentrations of e.g. 25 μM (see page 104, right column, lines 24-32). For the sake of completeness we would like to mention that the concentrations used in our experimental report D12 are 3, 10 and 30 μM.

Document D9 can therefore not support the allegation that the whole claimed targeting area is suitable.

It has furthermore been alleged that the authors of D9 who belong to the same research group as the present inventors have confirmed that the claimed target region is an advantageous target for exon 53 skipping. It should be considered that the authors of D9 belong to the same research group as the inventors of the present application. Therefore, the persons are either identical or at least well connected in a research network. It is also not proven that the disclosure of the present application inspired any skilled person outside the group of the inventors of the present patent.

A careful analysis of document D9 makes plainly evident that the conclusions drawn by patentee's representatives are not supported by the experimental evidence published in D9. Therefore, D9 is not relevant for two reasons, namely first, it is in general not admissible to use the post-published document as support for the presence of an inventive step and second, even if it was considered to be admissible the arguments of patentee are not supported by the facts published in D9.

1.3.2 Length and chemical backbone of oligonucleotide

Under section 6.1 (page 9) patentee stressed the fact that the oligonucleotide h53AON1 is outside of granted claim 1 due to (a) its length and (b) its chemical backbone. Patentee argued that the 18-mer AON of D3 fails to comply with the claimed requirements of being 20-31-mer and being PMO.

The Opposition Division correctly considered document D3 as the closest prior art. Uncontestably D3 discloses the oligonucleotide h53AON (page 48) having a length of 18 nucleotides. Document D3 is, however, not restricted to the disclosure of these single oligonucleotides in the examples. Claim 3 mentions that the oligonucleotide may have 14 to 50 nucleotides. In claim 17, 16 to 80 nucleotides are mentioned and on page 9, lines 27-30 it is disclosed that the oligonucleotides have preferably a length between 16 and 50 nucleotides. Therefore, D3 provides for the person skilled in the art the suggestion to use oligonucleotides which are longer.

D3 discloses also that the oligonucleotide may be a morpholino derivative. In this respect we refer to claim 12 and page 10, lines 14-16.

We agree therefore with the conclusion of the Examining Division regarding D3. If D3 is considered to be the closest prior art, the claimed subject matter is rendered obvious by D3 itself since D3 does not only disclose the highly relevant oligonucleotide h53AON but also that longer oligonucleotides are preferred and that the morpholino modification is preferred.

Furthermore, patentee argued (page 10) that D3 states that there may be additional nucleotides added to the 18-mer whereby, however, such additional nucleotides to be added do not contribute to hybridization by referring to the following statements of D3:

> *As mentioned, oligonucleotides of the invention* ***do not have to consist of only oligonucleotides that contribute to hybridisation*** *to the targeted exon.* ***There may be additional material and/or nucleotides added****.* [emphasis added]

First, patentee's argument is based on a misleading interpretation of the quoted sentence. The sentence says that in general added nucleotides may contribute to hybridization. In the exception there may also be additional nucleotides which do not contribute to hybridization. Regularly the oligonucleotides consist only of nucleotides which contribute to hybridization and are therefore complementary to the target sequence.

Second, we would like to submit that even if we followed patentee's interpretation for the sake of argument, the quoted sentence does not contradict our position. When the 18-mer of D3 is extended to 20-mer by adding two extra nucleotides, the 20-mer would have at least 90% sequence identity to the target sequence regardless of the base type of the two extra nucleotides since at least 18 out of 20 nucleotides would perfectly match the target sequence, such oligonucleotides might be considered as "specifically hybridizable".

Patentee's argument must be understood with the definition of "specifically hybridizable" provided in the present specification. The present specification states that "[i]t is understood in the art that the sequence of an antisense molecule need not be 100% complementary to that of its target sequence to be specifically hybridisable." (see paragraph 0053).

Patentee's counterargument is therefore not convincing. If the quoted passage is interpreted in a literal sense, the additional nucleotides are complementary to the target sequence. If, however, we follow for the sake of argument patentee's argument, namely that the additional nucleotides are not complementary to the target sequence, such a sequence would nevertheless fall under the claim since the claim does not require a 100% complementary in view of the feature "specifically hybridizable". Therefore, patentee's counterargument is groundless.

1.3.3 Does the claimed subject matter show a superior effect over the whole breadth of the claim?

1.3.3.1 D12 (patentee's experiments)

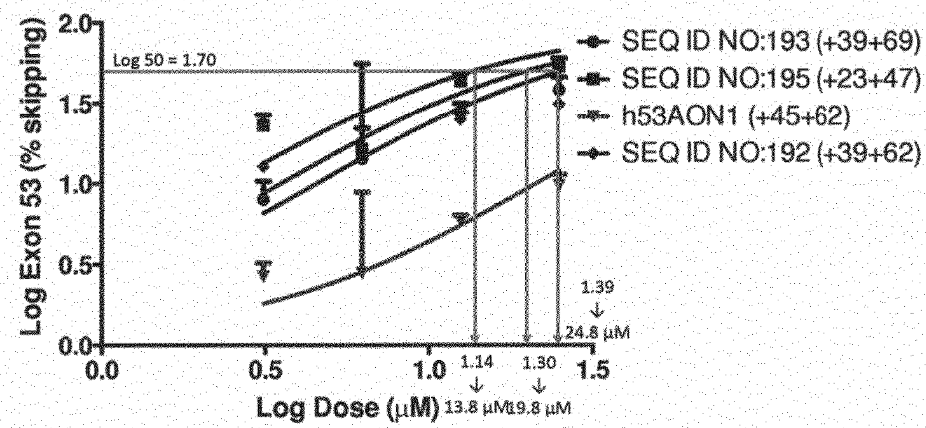
The experimental data of D12 as submitted by the patentee shall allegedly demonstrate that the AON of the opposed patent has higher activity than the 18-mer of D3. We do not agree.

The OD has acknowledged that AONs tested in D12 are of 24 to 31-mer sizes, and that whether smaller AONs of 20 to 21-mer sizes would have higher activities than those of 18-mer size cannot be appreciated from the results of D12. We agree to the OD's opinions on this point since there is no evidence on file to support that 20-23 mer oligonucleotides are more efficient than the prior art oligonucleotides or even equivalents.

Furthermore, the results as presented in D12 are not correct since the numerical values indicated as EC50 seem to have been incorrectly calculated.

RD Cells (page 2 of D12)

	EC50	
SEQ ID NO:193 (+39+69)	4.536	24.8
SEQ ID NO:195 (+23+47)	2.431	13.8
h53AON1 (+45+62)	21.25	-
SEQ ID NO:192 (+39+62)	3.531	19.0



The Opponent has calculated as schematically shown above the correct EC50 values for the RD cell data of D12, which are shown in the table below.

Correct EC50 values for RD cells

	(1) EC50 shown in D12(=EC10) (μM)	(2) Correct EC50 (μM)	Fold (=(2)/(1))
+39+69	4.536	**24.8**	5.47
+23+47	2.431	**13.8**	5.68
+39+62	3.531	**19**	5.38

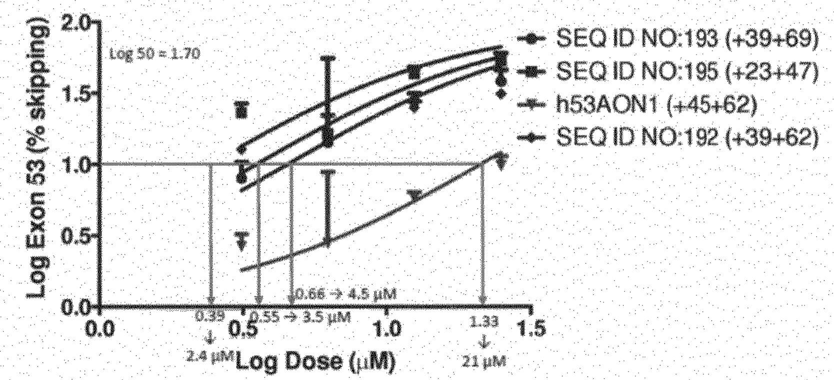
<u>The EC50 value for h53AON1(+45+62) was not available as the skipping activity was below 50%</u>. For the same reasons, the EC50 values for any of the AONs in myoblast were not available either.

The numerical values indicated in D12 as “EC50” are in fact those for EC10. EC10 can be obtained at intersection points of the horizontal line at 1.0 (=log10) of the vertical axis, i.e. “Log Exon 53 (% skipping)”, and the curve for each sample.

The "EC50" value for SEQ ID NO 193 in RD cells as indicated in the table of D12 is 4.536. The common logarithm of 4.536 (=log4.536) equals to 0.6567. This is exactly the value of the intersection between the line at 1.0 of the vertical axis (=EC10) and the curve for SEQ ID NO:193.

RD Cells

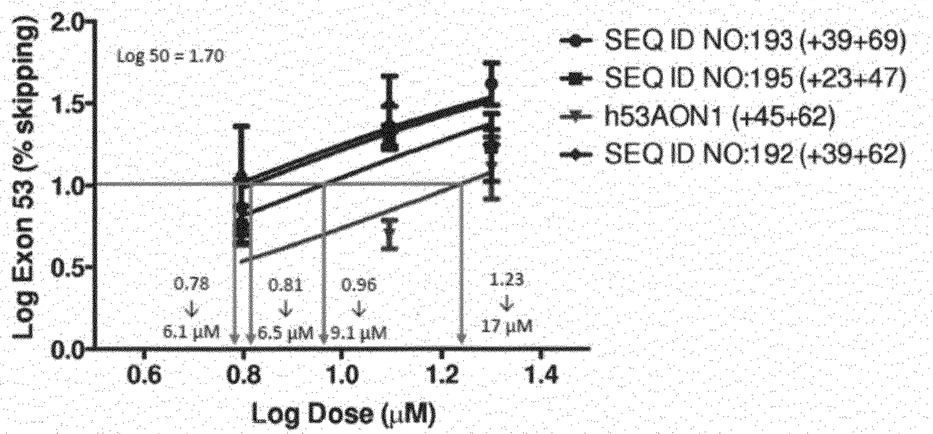
	EC50
SEQ ID NO:193 (+39+69)	4.536
SEQ ID NO:195 (+23+47)	2.431
h53AON1 (+45+62)	21.25
SEQ ID NO:192 (+39+62)	3.531



In analogous manner the values were recalculated for the experiments performed with myoblasts:

Myoblasts

	EC50
SEQ ID NO:193 (+39+69)	6.482
SEQ ID NO:195 (+23+47)	9.135
h53AON1 (+45+62)	17.13
SEQ ID NO:192 (+39+62)	6.061



Therefore, Patentee's arguments based on D12 cannot support the presence of an inventive step since the calculations are incorrect.

1.3.3.2 (D8)

Patentee criticizes in the submission of February 22, 2017 on page 13 that opponent has deliberately selected two sequences which significantly overlap and allegedly anneal to very similar regions within the annealing sites. It should be mentioned, however, that this is no valid counterargument since patentee has the burden to show that an alleged superior effect is obtainable over the whole scope of the claim (T 939/92).

It is true that opponent filed experimental evidence as report D8 using AONs targeting +45+62, +49+69 and +50+69. The sequences tested, in particular +49+69 and +50+69, are similar.

In addition, in the results D8 are criticized since the activities of the same AON change from the best to worst depending on the concentration.

Against this allegation, the Opponent wishes to point out that patentees allegation is in the same manner applicable to the data of D12. In the results of myoblasts, SEQ ID NO:192 (+39+62) achieves the highest exon skipping at the concentration of 0.8 (= 6.3 µM) whereas SEQ ID NO:193 (+39+69) achieves the highest at the concentration of 1.3 (=20 µM). In addition, in the result of RD cells of D12, SEQ ID NO:192 (+39+62) achieves the second highest exon skipping at the concentration of 0.5 (= 3 µM) whereas it achieves the third highest at 1.4 (= 24 µM).

Furthermore, it has been objected that controls are not present in D8. It seems, however, that also D12 does not contain controls.

In view of this objection opponent hereby submits an amended version of D8, i.e. D8-1, where the control of AON targeting the 39th to 69th nucleotides in exon 53 designated as "SEQ ID NO: 4" is added. In comparison to the control, the faintness of the skipping activities for SEQ ID NOs: 2 and 3 is further highlighted. In addition, the data for 3µM is deleted in D8-1, because it turned out that a concentration of 3 µM as presented in the original measurements (D8) was too low.

In addition, Opponent hereby submits another experimental report as D13. In D13, an extra set of AONs encompassed by claim 1 of the present patent is tested, namely SEQ ID NOs: 5 to 10. SEQ ID NOs: 1 and 4 are identical to those of D8-1, which are h53AON1 and +39+69, respectively. As can be seen in the table in "Results" of D13, it is clearly demonstrated that all of SEQ ID NOs: 5 to 10 have very faint skipping activities. A note should be made that the skipping activities for SEQ ID NOs: 5 to 10 do not escalate in dose dependent manners. Rather the skipping activities for SEQ ID NOs: 5 to 10 stays near 2.0% to 3.0% regardless of the doses.

At least based on D8-1, a person of skill in the art would undoubtedly understand that the patented invention fails to provide its intended effect over the entire scope of the claim. When considering the results shown in D13, said understanding will further be confirmed.

1.3.4 Summary regarding Art. 56 EPC

Regarding Article 56 EPC we agree with the preliminary non-binding finding of the Opposition Division that the claimed subject matter is not based on an inventive step.

II. Auxiliary Request 1

The Auxiliary Request 1 differs from the Main Request insofar that granted claims 4 and 5 are deleted. We agree with the preliminary opinion of the Opposition Division that Auxiliary Request 1 is not admissible in view of a violation of Rule 80.

Since claim 1 of Auxiliary Request 1 corresponds exactly to claim 1 of the Main Request our objections as raised for the Main Request apply also to Auxiliary Request 1.

III. Auxiliary Request 2

In claim 1 of the Auxiliary Request 2 the term "or both" has been deleted and the two annealing sites are connected by the conjunction "or".

Our objections as raised with regard to the Main Request apply also to Auxiliary Request 2 with the proviso that the objections relating to Article 123(2) EPC concerning "both" are not maintained for Auxiliary Request 2.

IV. Auxiliary Request 3

The objections as raised with regard to the Main Request are maintained with the proviso that specific objections relating to the annealing site H53A(+23+47) and to "or both" are not maintained.

V. Auxiliary Request 4

This seems to be a combination of the amendments proposed in Auxiliary Request 1 and Auxiliary Request 2.

In claim 1 of Auxiliary Request 4 the term "or both" has been deleted and the two annealing sites are connected by the conjunction "or".

The Auxiliary Request 4 differs from the Main Request insofar that granted claims 4 and 5 are deleted. We agree with the preliminary opinion of the Opposition Division that Auxiliary Request 4 is not admissible in view of a violation of Rule 80.

Since claim 1 of Auxiliary Request 4 corresponds exactly to claim 1 of the Auxiliary Request 2 our objections as raised for the Main Request apply also to Auxiliary Request 4 whereby our objections concerning "both" are no longer applicable.

VI. Auxiliary Request 5

This seems to be a combination of the proposed amendments as presented for Auxiliary Requests 1 and 3.

The objections as raised with regard to the Main Request are maintained with the proviso that specific objections relating to the annealing site H53A(+23+47) and to "or both" are not maintained.

Auxiliary Request 5 further differs from the Main Request insofar that granted claims 4 and 5 are deleted. We agree with the preliminary opinion of the Opposition Division that Auxiliary Request 5 is not admissible in view of a violation of Rule 80.

VII. Conclusion

Since there is no set of claims on file which complies with the requirements of the EPC the patent has to be revoked.

Dr. G. Keller

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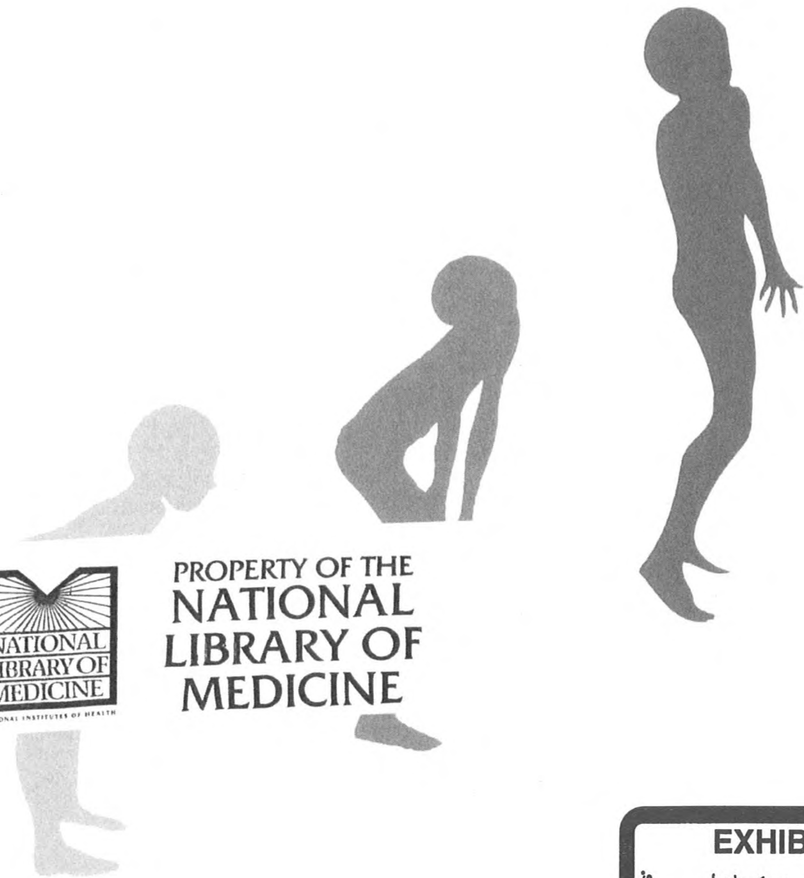
20 (2) 95–154
ISSN 0960-8966

Neuromuscular Disorders



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Neuromuscular Disorders

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Volume 20 No. 2 (2010)

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Publication information: *Neuromuscular Disorders* (ISSN 0960-8966). For 2010 Volume 20 is scheduled for publication. Subscription prices are available upon request from the Publisher. Subscriptions are accepted on a prepaid basis only and are entered on a calendar year basis. Issues are sent by surface mail except to the following countries where air delivery by SAL mail is ensured: Argentina, Australia, Brazil, Canada, Hong Kong, India, Israel, Japan, Malaysia, Mexico, New Zealand, Pakistan, PR China, Singapore, South Africa, South Korea, Taiwan, Thailand, USA. For all other countries airmail rates are available upon request. Claims for missing issues should be make within six months of our publication (mailing) date.

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Neuromuscular Disorders 20 (2010) 102–110

Contents lists available at ScienceDirect

Neuromuscular Disorders

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Comparative analysis of antisense oligonucleotide sequences targeting exon 53 of the human *DMD* gene: Implications for future clinical trials

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ARTICLE INFO

Article history:
Received 13 July 2009
Received in revised form 20 October 2009
Accepted 27 October 2009

Keywords:
Exon skipping
Duchenne muscular dystrophy
Antisense oligonucleotides
Phosphorodiamidate morpholino oligomer

ABSTRACT

Duchenne muscular dystrophy (DMD) is caused by the lack of functional dystrophin protein, most commonly as a result of a range of out-of-frame mutations in the *DMD* gene. Modulation of pre-mRNA splicing with antisense oligonucleotides (AOs) to restore the reading frame has been demonstrated in vitro and in vivo, such that truncated but functional dystrophin is expressed. AO-induced skipping of exon 51 of the *DMD* gene, which could treat 13% of DMD patients, has now progressed to clinical trials. We describe here the methodical, cooperative comparison, in vitro (in DMD cells) and in vivo (in a transgenic mouse expressing human dystrophin), of 24 AOs of the phosphorodiamidate morpholino oligomer (PMO) chemistry designed to target exon 53 of the *DMD* gene, skipping of which could be potentially applicable to 8% of patients. A number of the PMOs tested should be considered worthy of development for clinical trial.

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe muscle-wasting disease, affecting 1:3500 live male births, caused by the lack of functional dystrophin protein in skeletal muscles, as a result of frame-disrupting deletions or duplications or, less commonly, nonsense or missense mutations in the *DMD* gene [1]. Mutations that maintain the reading frame of the gene and allow expression of semi-functional, but internally-deleted dystrophin are generally associated with the less severe Becker muscular dystrophy (BMD) [1,2].

Transforming an out-of-frame DMD mutation into its in-frame BMD counterpart with antisense oligonucleotides (AOs) is the basis of the potentially exciting exon skipping therapy for DMD (reviewed by Muntoni and Wells) [3]. The hybridization of AOs to specific RNA sequence motifs prevents assembly of the spliceosome, so that it is unable to recognise the target exon(s) in the pre-mRNA and include them in the mature gene transcript [4,5]. AOs have been used to induce skipping of specific exons such that the reading frame is restored and truncated dystrophin expressed in vitro in DMD patient cells [6,5,7–9], and in animal models of the disease in vivo [4,10–13].

Initial proof-of-principle clinical trials, using two different AO chemistries (phosphorothioate-linked 2′-*O*-methyl modified bases (2′OMePS) [14] and phosphorodiamidate morpholino oligomer (PMO) [15]) for the targeted skipping of exon 51 of the *DMD* gene after intramuscular injection, have been performed recently with encouraging results. While both chemistries have excellent safety profiles [16,17], PMOs appear to produce more consistent and sustained exon skipping in the *mdx* mouse model of DMD [18–20], in human muscle explants [21], and dystrophic canine muscle cells in vitro [22]. However, for some human exons, 2′OMePS and PMO AONs performed equally well [17]. Since the mutations that cause DMD are so diverse, of those DMD patients with genomic deletions, skipping of exon 51 would have the potential to treat only 13% of such patients on the Leiden DMD database [23], and 15% of such patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO). Although any predictions on the frequency of mutations and percentage of skippable patients should be viewed with caution, it is undeniable that the continued development and analysis of AOs for the targeting of other DMD exons is vital.

Here we report the comparative analysis of a series of PMOs targeted to exon 53, skipping of which would have the potential to

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doi:10.1016/j.nmd.2009.10.013

treat a further 8% of DMD patients with genomic deletions on the Leiden database [23], and a further 13.5% of patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO). PMOs designed and previously tested in normal human skeletal muscle cells (hSkMCs) for the targeting of exon 53 [24] were further studied here in cells from a DMD patient with a relevant deletion (del 45–52). These PMOs were directly compared to a PMO based on a AO previously identified as being the most bioactive by Wilton et al. [25]. Time-course studies were performed to evaluate the persistence of skipping and dose-responses were examined. Findings from these experiments were supported by in vivo studies in a mouse model transgenic for the entire human dystrophin locus [12]. Collectively, this work reports a number of PMOs able to produce targeted skipping of exon 53 to levels that would suggest them worthy of consideration for upcoming PMO clinical trials.

2. Materials and methods

2.1. AO design

All AOs were synthesized as phosphorodiamidate morpholino oligomers (PMOs) by Gene Tools LLC (Philomath OR, USA).

2.2. DMD patient primary myoblast culture

Skeletal muscle biopsy samples were taken from a diagnostic biopsy of the quadriceps from a DMD patient with a deletion of exons 45–52. Informed consent was obtained before any processing of samples, and all work was carried out with the approval of the institutional ethics committee. Muscle precursor cells were prepared from the biopsy sample by sharp dissection into 1 mm^3 pieces and disaggregated in solution containing HEPES (7.2 mg/ml), NaCl (7.6 mg/ml), KCl (0.224 mg/ml), glucose (2 mg/ml), Phenol red (1.1 μg/ml), 0.05% Trypsin–0.02% EDTA (Invitrogen, Paisley, UK) in distilled water, three times at 37 °C for 15 min in Wheaton flasks with vigorous stirring. Isolated cells were plated in non-coated plastic flasks and cultured in Skeletal Muscle Growth Media (Promocell, Heidelberg, Germany) supplemented with 10% Foetal Bovine Serum (PAA Laboratories, Yeovil, UK), 4 mM L-glutamine and 5 μg/ml gentamycin (Sigma–Aldrich, Poole, UK) at 37 °C in 5% CO_2.

2.3. Nucleofection of DMD primary myoblasts

Between 2×10^5 and 1×10^6 cells/ml were pelleted and resuspended in 100 μl of solution V (Amaxa Biosystems, Cologne, Germany). The appropriate PMO to skip exon 53 was added to the cuvette provided, sufficient to give the concentrations described, followed by the cell suspension, and nucleofected using the Amaxa nucleofector 2, program B32. Five hundred microliters of medium was added to the cuvette immediately following nucleofection [26]. This suspension was transferred to a 6 well plate in differentiation medium. Nucleofected cells were maintained in differentiation media for 3–21 days post treatment before extraction of RNA or protein. Transfections were performed blindly and in each experiment in triplicate. Each experiment was repeated at least once to ensure reproducibility of results.

2.4. Lactate dehydrogenase cytotoxicity assay

A sample of medium was taken 24 h post-transfection to assess cytotoxicity by release of lactate dehydrogenase (LDH) into the medium, using the LDH Cytotoxicity Detection Kit (Roche, Burgess Hill, UK), following the manufacturer's instructions. The mean of three readings for each sample was recorded, with medium only, untreated and dead controls. The readings were normalised for background (minus medium only) and percentage toxicity expressed as [(sample-untreated)/(dead-untreated) × 100].

2.5. Transgenic human DMD mice

A transgenic mouse expressing a complete copy of the human *DMD* gene has been generated [12,27]. Experiments were performed at the Leiden University Medical Center, with the authorization of the Animal Experimental Commission (UDEC) of the Medical Faculty of Leiden University as described previously [9]. Twenty micrograms of each PMO was injected once into two gastrocnemius muscles, pretreated with cardiotoxin. Mice were sacrificed 1 week after the injection, and RNA harvested from the isolated gastrocnemius muscles and analysed by RT-PCR.

2.6. RNA isolation and reverse transcription-polymerase chain reaction analysis

RNA was isolated and analysed by RT-PCR, as described previously [9]. Primer sequences and detailed PCR protocols are available on request. PCR products were analysed on 1.5% (w/v) agarose gels in Tris–borate/EDTA buffer. Skipping efficiencies were determined from gel images by comparing induced shortened dystrophin mRNAs to the intact transcript of the full length using densitometric analysis with Image J software (for patient samples) or by quantifying the skipped products with DNA 1000 LabChip Kit on the Agilent 2100 bioanalyzer (Agilent Technologies, USA) (for hDMD mouse samples). Skipping percentages were calculated as the amount of skip transcripts relative to the total transcripts (skip and full length). Equal amounts of the induced and intact transcripts would be regarded as representing 50% efficiency, while an estimate of 25% exon skipping would be represented by the intact transcript being three times more abundant than the band representing the induced transcript. Likewise, if the induced transcript was present at three times the level of the intact transcript, the exon skipping efficiency would be assessed to be 75%. Where appropriate, the two-tailed student's *t*-test was used to determine the statistical strength of the skipping efficiencies produced.

2.7. Sequence analysis

RT-PCR products were excised from agarose gels and extracted with a QIAquick gel extraction kit (Qiagen, Crawley, UK). Direct DNA sequencing was carried out by the MRC Genomics Core Facility.

2.8. Western blot analysis of dystrophin protein

DMD patient cells, transfected as described and cultured in differentiation medium, were harvested 7, 14 or 21 days post-transfection. Cells (4×10^5) were pelleted and resuspended in 50 μl of loading buffer (75 mM Tris–HCl pH 6.8, 15% sodium dodecyl sulphate, 5% ß-mercaptoethanol, 2% glycerol, 0.5% bromophenol blue and complete mini protease inhibitor tablet). Samples were incubated at 95 °C for 5 min and centrifuged at 18,000*g* for 5 min. Twenty microliters of sample was loaded per well in a 6% polyacrylamide gel with 4% stacking gel. Protein from CHQ5B cells differentiated for 7 days was used as a positive control for dystrophin. Gels were electrophoresed for 5 h at 100 V before blotting on nitrocellulose membrane at 200 mA overnight on ice. Blots were stained with protogold to assess protein loading, then blocked in 10% nonfat milk in PBS with 2% Tween (PBST) for 3 h. Blots were probed with antibodies to dystrophin, NCL-DYS1 (Vector Labs, Peterborough, UK) diluted 1:40 and to dysferlin, Hamlet1 (Vector Labs)

diluted 1:300 in 3% non-fat milk/PBST. An anti-mouse, biotinylated secondary antibody (diluted 1:2000; GE Healthcare, Amersham, UK) and streptavidin/horse radish peroxidise conjugated antibody (1:10,000; Dako, Ely, UK) allowed visualisation in a luminol-HRP chemiluminescence reaction (ECL-Plus; GE Healthcare) on Hyperfilm (GE Healthcare), exposed at intervals from 10 s to 4 min.

2.9. Statistical analysis

For the blind comparison at 300 nM in DMD patient cells, data from two separate experiments performed in duplicate and triplicate respectively were pooled and compared by two-tailed student *t*-test. Dose–response and time-course experiments were compared by two-tailed, paired *t*-test.

3. Results

Twenty-four AOs designed to target exon 53 of the *DMD* gene have been previously tested in normal human skeletal muscle cells (hSkMCs) [24,25]. Table 1 summarises the names and target sequence characteristics of these AOs (shown in bold), and % skipping produced by each in normal hSkMCs. However, studies in normal hSkMCs are limited as they do not allow assessment of the therapeutic effect at the protein level (i.e. dystrophin restoration). Further studies have therefore been performed here to elucidate and confirm which AO(s) would have the potential as a treatment for patients with an eligible deletion. AOs, whose target sites are within the sequence +29 to +74 of exon 53, the region previously shown to be in open conformation, binding to which interferes with spliceosome-mediated pre-mRNA splicing, such that exon 53 is skipped [24,25], were directly compared in exon 53-skippable patient cells (at UCL), and in the humanised DMD (hDMD) mouse (at LUMC). The AOs were all synthesized as PMOs to allow direct comparison of skipping efficacy. While PMOs were hybridized to mixed-backbone DNA leashes in the previous study [24], the nucleofection method used here was performed on unleashed PMOs.

3.1. Comparison of PMOs to exon 53 in DMD patient cells

Our comparative evaluation of PMO-induced exon skipping efficiencies was performed in a blinded fashion. All transfections were performed in triplicate and repeated at least once to ensure uniformity of results. Skipping efficiencies were determined from RT-PCR gel images by comparing induced shortened dystrophin mRNAs to the intact full length transcript using densitometric analysis, as described previously [25]. Sequencing of RT-PCR products confirmed the targeted skipping of exon 53 (results not shown). For quantification, the skip-products were analysed using densitometric analysis with Image J software. This technique for quantifying skipping efficiencies of AOs targeted to the *DMD* gene has been published previously [9,17]. Real-time PCR quantification of intact and induced transcripts has proven to be impossible due to a number of obstacles (variation of amplification efficiencies of each transcript, possible interference of intact and induced transcript primers/probes with each other) (results not shown). No DMD exon skipping studies thus far reported have included real-time PCR quantification of AO efficacy, and we believe we have used the best method available for quantification. Skipping efficiency is given as the percentage of skip transcript over the total amount of transcript (skip and full length). AOs were sub-divided on the basis of their skipping efficiency. PMOs that produced over 50% exon skipping were designated as Type 1, those that produced between 25% and 50% exon skipping were described as Type 2, while those that produced less than 25% as Type 3. Where appropriate, the two-tailed student's *t*-test was used to assess significant differences between AOs.

The 13 PMOs, whose target sites are within the sequence +29 to +74 of exon 53, were compared directly at a 300 nM dose by nucleofection [26]. This dose was selected for comparison, since such concentrations of AOs have been used in numerous previous exon skipping studies in DMD [5,6,9]. PMOs-G, -H and -A were the most efficient, producing a mean of 73% (±4.10%), 68% (±4.77%) and 68% (±4.14%) exon skipping respectively (classified as Type 1) (Fig. 1). The other PMOs tested produced the following exon skipping levels: PMO-I, 63% (±7.5%); PMO-B, 56% (±6.29%); PMO-M, 52% (±10.78%) (all classified as Type 1); PMO-J, 37% (±4.95%); (classified as Type 2). All other PMOs tested gave exon skipping at levels of between 15% and 26%. When compared by two-tailed student *t*-test, PMO-G (the most efficient PMO) gave significantly higher levels of exon skipping than PMOs -C ($p < 0.0001$), -D ($p < 0.0001$), -E ($p < 0.0001$), -F ($p < 0.0001$), -J ($p = 0.0005$), -K ($p = 0.0002$) and -L ($p < 0.0001$), but was not significantly more effective than the other PMOs tested. The more efficacious PMOs should produce sustained and pronounced exon skipping when applied at lower concentrations. Therefore, the six most effective PMOs (i.e. Type 1) (-A, -B, -G, -H, -I and -M) were selected for dose–response and time-course experiments.

When the concentration dependence of exon skipping was examined for the most efficient PMOs, skipping levels approaching 30% were evident for the Type 1 PMOs -G and -H at concentrations as low as 25 nM (Fig. 2a, b). The other PMOs classified as Type 1 (PMOs -A, -B, -I and -M) did not induce such levels of exon skipping when used at lower concentrations. Similar levels of skipping (30%) were only achieved by PMO-A, PMO-B and PMO-M at 100 nM, while PMO-I needed to be present at 200 nM to produce over 30% exon skipping (Fig. 2a, b). This is why the concentration dependence of exon skipping is a valuable tool in ascertaining the most efficient AO(s).

The exon skipping produced by the six Type 1 PMOs was shown to be persistent, lasting for up to 10 days after transfection, with over 60% exon skipping observed for the lifetime of the cultures for PMOs -A, PMO-G and PMO-H (Fig. 3a, b). When compared by two-tailed, paired *t*-test across all time-points, PMO-G gave significantly higher levels of skipping than PMOs -B, -H, -I and -M ($p = 0.0004$, 0.0126, 0.0008 and 0.0004, respectively) and bordered on significance for PMO-A ($p = 0.0550$). Three of the most effective Type 1 PMOs (-A, -G and -H) were also compared in a longer time-course experiment up to 21 days after transfection (Fig. 3c, d). PMO-G gave sustained high levels of exon skipping (over 60%) for the 21 days whereas skipping by PMOs -A and -H had fallen to 48% and 46%, respectively by day 17. When compared by two-tailed, paired *t*-test across all time-points, PMO-G gave significantly higher levels of skipping than PMOs -A and -H ($p = 0.0422$ and 0.0231, respectively). There was no evidence that any of the PMOs tested caused cellular cytotoxicity relative to mock-transfected controls, as assessed by visual inspection, and lactate dehydrogenase release into culture medium (results not shown). The relative efficacy of the six Type 1 PMOs in the direct comparison, dose–response and time-course assays is summarised in Fig. 4a. Exon 53 skipping by PMO-G is used as the baseline set at 100%. This clearly shows the PMO-G outperforms the other Type 1 PMOs in vitro in patient cells. However, it should also be noted that a number of the other Type 1 PMOs, namely PMO-A and PMO-H, also appear to perform very well across these three comparative tests.

The most effective Type 1 PMO (PMO-G) from the time-course experiments was compared to the most effective PMO to skip exon 51 that has been taken forward to clinical trial, in DMD patient cells with an amenable mutation (del 45–52 for exon 53 skipping and del 48–50 for exon 51 skipping) in a dose–response experiment from 25 to 400 nM (see Fig. 4b). PMO-G gave higher levels

Table 1
Table summarising the characteristics of PMOs used. Characteristics of the PMOs and their target sites listed (taken from [24]).

	PMO	% Skip	Position Start	Position End	%GC	Exon-PMO binding energy	PMO-PMO binding energy	% Open[a]	Ends in open loops[a]	% Overlap with hybrid. peak
(a)										
A	h53A1	12.7	+35	+59	52	−38.6	−17.4	50	2	92
B	h53A2	9.7	+38	+62	56	−36.1	−17.4	46.7	1	100
C	h53A3	2.0	+41	+65	56	−36.7	−13.7	36.7	0	0
D	h53A4	10.5	+44	+68	48	−34.3	−8.5	20	0	100
E	h53A5	9.0	+47	+71	48	−35.5	−8.5	43.3	2	100
F	h53A6	0.3	+50	+74	48	−35.3	−8.5	43.3	2	92
N	h53B1	0	+69	+93	28	−22.1	−12.1	53.3	1	0
O	h53B2	0.6	+80	+104	48	−30.1	−11.3	23.3	1	0
P	h53B3	3.0	+90	+114	48	−34.5	−5.5	48	2	0
Q	h53C1	0	+109	+133	48	−32.4	−9.8	46.7	2	0
R	h53C2	0	+116	+140	56	−31.3	12.7	33.3	1	0
S	h53C3	0	+128	+152	60	−34.6	−13.7	26.7	1	0
T	h53D1	0	+149	+173	52	−34.1	−13.4	30	1	0
U	h53D2	0.9	+158	+182	48	−36.5	−14.5	40	2	0
V	h53D3	3.7	+170	+194	36	−34.3	−11.2	40	1	0
W	h53D4	12.3	+182	+206	32	−30.9	−9.2	63.3	1	0
X	h53D5	7.9	+188	+212	36	−31.5	−3.3	66.7	1	0
G	h53A30/1	52.4	+30	+59	50	−48.1	−17.4	56.7	1	92
H	h53A30/2	87.2	+33	+62	53	−45.1	−17.4	63.3	1	100
I	h53A30/3	80.1	+36	+65	53	−44.6	−17.4	53.3	1	100
J	h53A30/4	38.6	+39	+68	50	−43.4	−17.4	43.3	1	100
K	h53A30/5	9.4	+42	+71	47	−42.4	−11.3	46.7	1	100
L	h53A30/6	35.9	+45	+74	47	−42.3	8.5	56.7	1	100
M	H53A	N/D	+39	+69	52	−48.5	−17.4	48.4	2	100

	PMO	# Rescue ESE sites	% Overlap with Rescue ESE	% Overlap with PESE	% Overlap with PESS	ESE finder values over threshold[b] SF2/ASF	BRCA1	SC35	SRp40	SRp55	Tra2B	9G8
(b)												
A	h53A1	7	56	84	0	6.58	7.26	0	3.12	0	24.04	19.02
B	h53A2	4	32	72	0	6.58	7.26	0	3.12	0	7.25	19.02
C	h53A3	3	32	60	0	6.58	7.26	0	3.12	0	7.25	11.9
D	h53A4	4	28	48	8	6.58	7.26	0	3.12	0	7.25	11.9
E	h53A5	3	36	36	20	6.58	7.26	0	3.12	0	7.25	11.9
F	h53A6	2	36	28	32	6.58	7.26	0	0	0	7.25	11.9
N	h53B1	5	56	40	40	0	9.26	3.62	10.66	0	5.06	1.1
O	h53B2	5	60	60	0	0	9.26	3.62	4.73	0	5.06	8.28
P	h53B3	8	72	64	0	3.49	9.26	3.44	4.73	0	24.04	28.68
Q	h53C1	6	52	72	0	4.19	6.72	0	2.04	0	24.04	28.68
R	h53C2	1	24	60	0	4.19	6.72	10.2	4.38	0	0	8.28
S	h53C3	1	24	32	0	3.49	6.41	10.2	4.38	6.86	0	14.18
T	h53D1	4	40	32	0	0.52	0	18.68	0	6.86	0	12.71
U	h53D2	6	44	32	0	0.52	1.8	18.68	0.42	0	0	12.71
V	h53D3	9	64	0	0	0	1.8	0	6.95	0	24.04	10.49
W	h53D4	16	96	24	0	8.5	11.95	0	7.67	0.33	24.04	7.14
X	h53D5	14	92	44	0	8.5	11.95	0	7.67	0.33	24.04	7.14
G	h53A30/1	9	60	86	0	6.58	7.26	0	3.12	0	24.04	19.02
H	h53A30/2	8	53	77	0	6.58	7.26	0	3.12	0	24.04	19.02
I	h53A30/3	6	43	67	0	6.58	7.26	0	3.12	0	24.04	19.02
J	h53A30/4	4	43	57	7	6.58	7.26	0	3.12	0	7.25	11.9
K	h53A30/5	5	47	47	17	6.58	7.26	0	3.12	0	7.25	11.9
L	h53A30/6	5	48	37	27	6.58	7.26	0	3.12	0	7.25	11.9
M	H53A	4	45	58	10	6.58	7.26	0	3.12	0	7.25	11.9

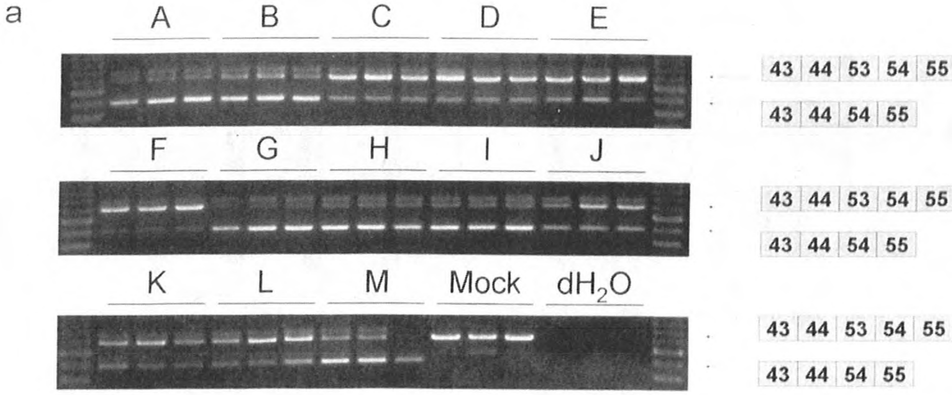
Alphabetical codes shown in bold represent the PMOs compared in this present study.
[a] Calculated as % of PMO target site in open structures on predicted RNA secondary structure obtained using MFOLD analysis. The position of the PMO target sites relative to open loops in the RNA secondary structure is listed (0, no ends in open loops; 1, one end in an open loop; 2, both ends in open loops).
[b] In our analyses, SR binding sites were predicted using splice sequence finder (http://www.umd.be/SSF/) software. Values above threshold are given for PMOs whose target sites cover 50% or more of potential binding sites for SF2/ASF, BRCA1, SC35, SRp40, SRp55, Tra2β and 9G8.

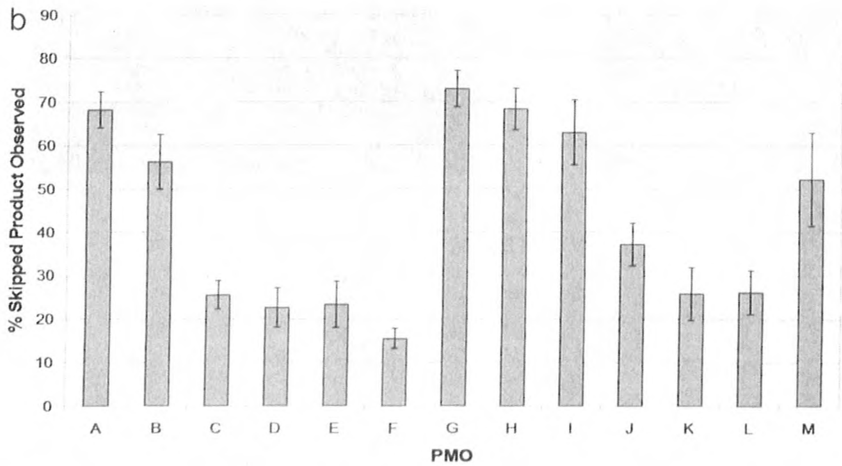
of skipping at all doses tested. When compared by two-tailed, paired *t*-test, PMO-G across all concentrations used, gave significantly higher levels of skipping than h51A ($p = 0.0033$).

Western blot analysis of DMD patient (del 45–52) cell lysates, treated in culture with the Type 1 25mers (PMOs -A and-B) and 30mers (-G, -H, -I and -M) is shown in Fig. 5a. De novo expression of dystrophin protein was evident with all six PMOs, but was most pronounced with PMOs -H, -I, -G and -A, producing 50%, 45%, 33% and 26% dystrophin expression, respectively, relative to the positive control, and seemingly weakest with PMO-B and PMO-M (11% and 17% dystrophin expression respectively, relative to the positive control). Although there are limitations to quantifying Western blots of this nature, the qualitative importance of the data holds.

3.2. Comparison of PMOs to exon 53 in humanised DMD mouse

The hDMD mouse is a valuable tool for studying the processing of the human *DMD* gene in vivo, and as such provides a model for studying the in vivo action of PMOs, prior to clinical testing in

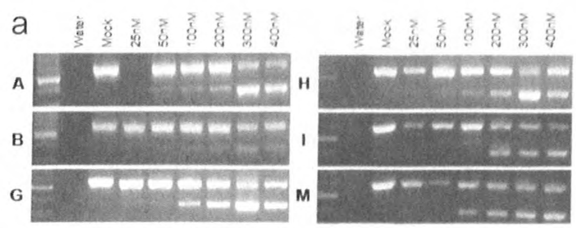


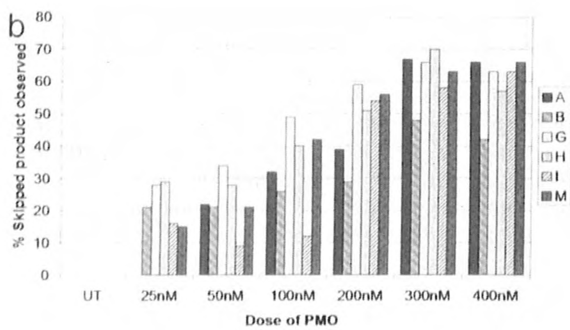


c

PMO	P-value
G vs A	0.4496
G vs B	0.0577
G vs C	<0.0001*
G vs D	<0.0001*
G vs E	<0.0001*
G vs F	<0.0001*
G vs H	0.4935
G vs I	0.2799
G vs J	0.0005*
G vs K	0.0002*
G vs L	<0.0001*
G vs M	0.1124

Fig. 1. Blind comparison of 13 PMO oligonucleotide sequences to skip human exon 53. Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected by nucleofection with each of the PMOs (300 nM) in triplicate. RNA was harvested 3 days following transfection, and amplified by nested RT-PCR. (a) Bars indicate the percentage of exon skipping achieved for each PMO, derived from Image J analysis of the electropherogram of the agarose gel (b). Skipped (477 bp) and unskipped (689 bp) products are shown schematically. The larger full length amplicon is often seen and is due to carry over of primers from the first round of the PCR into the second. (c) Efficacy of PMOs was compared by two-tailed, student t-test. PMO-G gave significantly higher efficacy of exon skipping than PMOs C, D, E, F, J, K and L ($^*p < 0.05$), but not significantly higher than PMOs A, B, H, I and M.

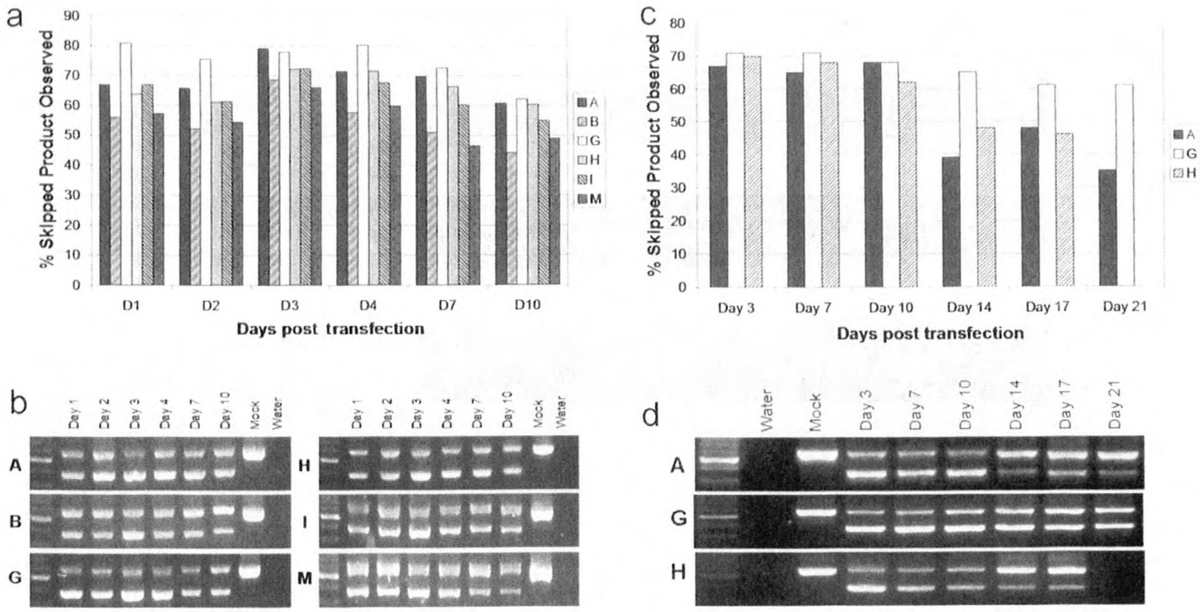




c

PMO	P-value
G vs A	0.0491*
G vs B	0.0022*
G vs H	0.0941
G vs I	0.0527
G vs M	0.0759

Fig. 2. Dose–response of the six best-performing PMOs. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected with the six best-performing PMOs by nucleofection, at doses ranging from 25 to 400 nM. RT-PCR products derived from RNA isolated from cells 3 days post-transfection were separated by agarose gel electrophoresis. (b) The percentage of exon skipping observed is expressed for each concentration of each PMO as a comparison of the percentage OD of skipped and unskipped band, as measured using Image J. (c) Data for each PMO over the range of doses were pooled and compared by two-tailed, paired student t-test. PMO-G gave significantly higher efficiency than PMOs A and B ($^*p < 0.05$) but did not give significantly higher efficacy than PMOs H, I and M.



e

PMO	P-value (10 days)	P-value (21 days)
G vs A	0.0550	0.0422*
G vs B	0.0004*	-
G vs H	0.0126*	0.0231*
G vs I	0.0008*	-
G vs M	0.0004*	-

Fig. 3. Persistence of dystrophin expression in DMD cells following PMO treatment. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45–52 were transfected by nucleofection with each of the six best-performing PMOs (300 nM), and were cultured for 1–10 days before extracting RNA. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (b). (c) Long term exon skipping up to 21 days after transfection with PMOs A, G and H. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (d). (e) Data for each PMO over the time-course experiments were pooled and compared by two-tailed, paired student t-test. Over the 10 day time-course experiment, PMO-G gave significantly higher efficiency than PMOs B, H, I and M ($^*p < 0.05$) but did not give significantly higher efficacy than PMO A. Over the 21 day time-course experiment, PMO-G gave significantly higher efficacy than PMOs A and H ($^*p < 0.05$).

patients. The Type 1 PMOs (-A, -G, -H, -I and -M) (20 μg) were injected into the gastrocnemius muscle of hDMD mice in duplo. RNA extracted from the muscles was analysed for exon 53 skipping by RT-PCR (Fig. 5b). For quantification, the skipped products were analysed using the DNA 1000 LabChip Kit on the Agilent 2100 bioanalyzer, which, unlike densitometry, corrects for fragment length. Skipping percentages were calculated as the amount of skip transcripts relative to the total transcripts (skipped and full length). Skipping of exon 53 was evident for each of the PMOs tested; average skipping seen in both legs was 8% for PMO-A, 7.6% for PMO-I, 7.2% for PMO-G, but a slightly lower level of 4.8% for PMO-H. PMO-M produced exon skipping levels of less than 1%, which is the detection threshold for the system used.

4. Discussion

We describe here the comparative analysis of PMOs designed to target exon 53 of the human *DMD* gene and thereby induce its skipping. Previously, a series of PMOs had been designed and their exon skipping efficacy investigated in normal human skeletal muscle cells [24]. These were directly compared to a PMO based on an AO previously identified as being the most bioactive by Wilton et al. [25]. Skipping efficiencies of the PMOs were compared here by two independent groups in two different systems (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [9]. The use of primary human myoblast cultures allowed controlled in vitro comparison of PMO sequences, and confirmation of skipping of exon 53 at the RNA level by PMOs in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent confirmation of optimal sequence(s) for the targeted skipping of exon 53.

The putative use of AOs to skip the exons which flank out-of-frame deletions is fast becoming a reality in the treatment of DMD boys. Indeed the restoration of dystrophin expression in the TA muscle of four patients, injected with a 2′OMePS AO optimised to target exon 51 of the *DMD* gene, has been reported recently [14]. Moreover a clinical trial using a PMO targeting exon 51 has recently been completed in seven DMD boys in the UK [15]. However, the targeted skipping of exon 51 would have the potential to treat only 13% of DMD patients on the Leiden database with genomic deletions [23]. There is therefore a definite requirement for the optimisation of AOs to target other exons commonly mutated in DMD. The targeted skipping of exon 53 of the human

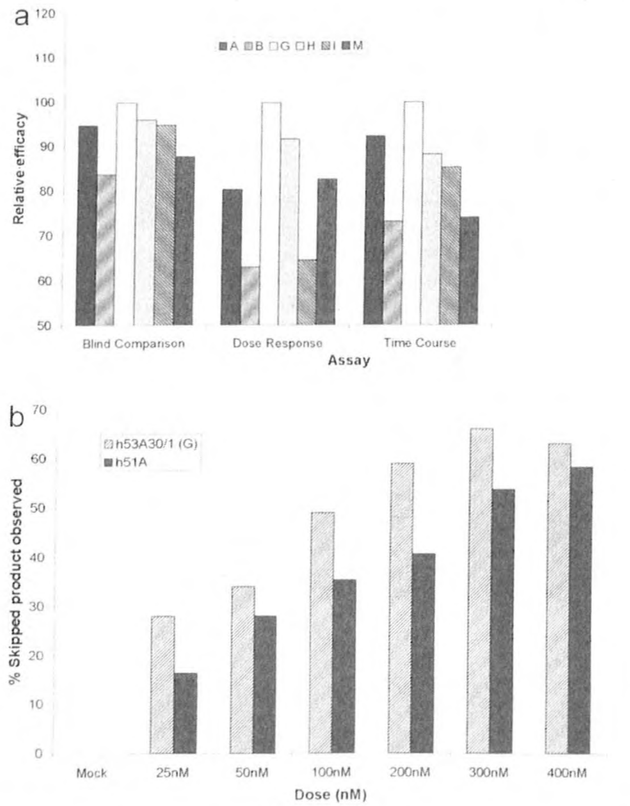


Fig. 4. Relative efficacy of Type 1 PMOs for the targeted skipping of exon 53. (a) The relative efficacy of the 6 most effective PMOs compared in the different assays. PMO-G was used as a baseline set at 100%. (b) The most effective PMO (G) compared to the most effective PMO to skip exon 51 in amenable DMD patients cells at doses ranging from 25 to 400 nM. PMO-G gave significantly higher levels of skipping ($p = 0.0033$ compared by two-tailed, paired t-test).

DMD gene would have the potential to treat a further 8% of DMD patients on the Leiden database [23], and 13.5% of patients on the UMD-DMD France mutations database (see http://www.umd.be/DMD/4ACTION/W_MONO).

There have been many large screens of AO bioactivity in vitro [24,25,28,29] which provide guidelines to aid AO design. The targeting of AOs to exonic splicing enhancer (ESE) motifs [25,28,29], RNA secondary structure, target site accessibility and strength of AO-target binding are all important predictors of AO efficacy [24,29]. Although there are tools available to aid the design of AOs for the targeted skipping of *DMD* exons, the empirical analysis of AOs is still required. Hence the importance of this study in the development of AO sequences as potential gene therapy drugs for DMD. The data presented here would indicate that PMOs targeting within the sequence +30+65 of exon 53 (namely PMO-A, -G and –H) produce levels of exon skipping that may be considered effective (over 50% exon skipping). There remains however the possibility that a stepped base-by-base screening of AOs across the entirety of exon 53 and some indeterminate distance into the flanking intronic sequences might reveal an AO with a better dose–response and longevity of action profile. Sequence +30+65 has been shown to be accessible to binding on hexamer hybridization array analysis and in open conformation by mfold prediction of pre-mRNA secondary structure [24]. These Type 1 PMOs can therefore bind more strongly since they can access their target site more directly. These thermodynamic considerations have also been reported in a complementary study of 2′OMePS AOs [29]. Indeed, the fact that the 30mer PMOs (-G, -H) were more bioactive than 25mer PMO counterpart (-A) targeted to the same open/accessible sites on the exon, would suggest that strength of binding of PMO to the target site may be the most important factor in determining PMO bioactivity. The influence of AO length on bioactivity has been reported elsewhere [9,30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart. Previous studies by the Leiden

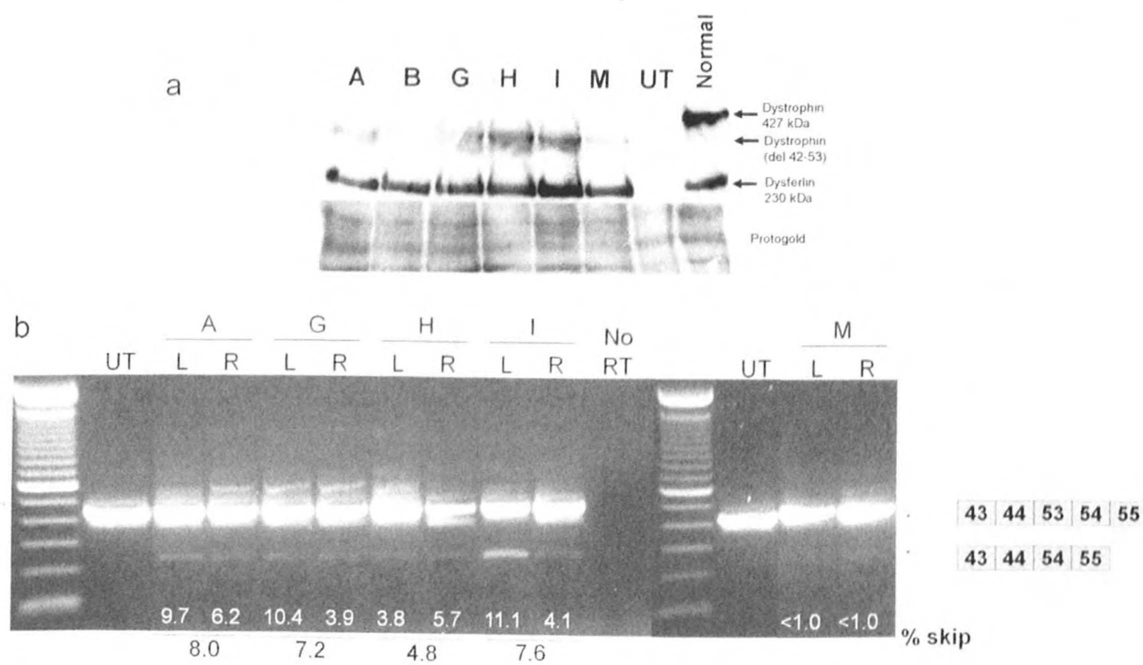


Fig. 5. Analysis of induced dystrophin protein expression in del DMD cells and in vivo efficacy of Type 1 PMOs in hDMD mice. (a) Western blot analysis was performed on total protein extracts from del 45–52 DMD cells 7 days after transfection with the six best PMOs (300 nM). Blots were probed with antibodies to dystrophin, to dysferlin as a muscle-specific loading control, and protogold for total protein loading control. CHQ5B myoblasts, after 7 days of differentiation were used as a positive control for dystrophin protein (normal). (b) PMOs (20 μg) were injected in a blind experiment into the gastrocnemius muscle of hDMD mice in duplo. RT-PCR analysis of RNA harvested from muscle isolated 1 week after injection was performed and products visualised by agarose gel electrophoresis. Quantification of PCR products was performed using a DNA LabChip.

group [28] suggest that the optimal 2′OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured control cells and 7% in the hDMD mouse. This 2′OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our PMOs produce higher levels of exon skipping could be a (combined) consequence of the different AO chemistries, length of AO used, type of cell used (patient vs. control) and the absolute target site of AO. No direct comparison was made here between PMO and 2′OMe AOs targeted to the same sequence of exon 53, since the purpose of this study was not to elucidate which chemistry was superior, but to ascertain the optimal target site for a PMO. A direct comparison would in fact be difficult as 2′OMe AOs are generally only 20 nucleotides long, whereas the PMOs used here were 25 and 30mers.

A PMO can be classified as an effective AO if it produces strong (over 50%), consistent and sustained exon skipping after administration to myogenic cells in vitro at low concentrations (25 nM). An AO reagent that produces sustained exon skipping at low concentrations would be preferable in the clinical setting as a gene therapy as this would lower the cost of treatment, extend the therapeutic action of exon skipping and reduce the possible toxicity of chronic AO administration. The levels of skipping produced in vitro by those PMOs targeting the sequence +30+65 are comparable, or indeed superior, to those reported pre-clinically for PRO051 [28] and AVI4658 [9], the 2′OMe and PMO AOs that are now both being used in Phase I/II clinical trials with encouraging results [14,15]. Indeed we directly demonstrate here the greater skipping efficacy of PMO-G relative to AVI4658 (H51A) over a range of concentrations (see Fig. 4b). However predicting the amount of skipping needed in vitro for an AO to be therapeutic in a patient is impossible; the efficiency of exon skipping is likely to differ from patient to patient and mutation to mutation, and the levels of dystrophin protein restoration will depend on the quality of the muscle itself when a clinical treatment is started.

When considering the data presented previously [24] and here as a whole, the superiority of the PMOs targeting the sequence +30+65 (i.e. PMOs -A, -B, -G and -H) is strongly indicated. The 30mers PMO-G and PMO-H produce higher levels of skipping relative to the 25mers PMO-A and PMO-B. In normal myoblasts, liposomal-carrier mediated transfection of leashed forms of these 30mer PMOs targeting produced over 50% skipping of exon 53, implying that they act extremely efficiently within the cell. This was confirmed in patient myoblasts using nucleofection as the entry method of naked PMOs into the cells. The different levels of exon skipping seen here in the patient cells relative to control cells is due to the different concentrations used (300 nM in patient cells versus 500 nM in normal cells), the different techniques used to introduce the PMOs into the cells, and the differences between the cells themselves. The dystrophin in patient cells is generally more readily skippable than normal cells [5,6,31]. The results seen in patient cells were reproducible, implying that sufficient PMO is getting into the cells to induce such skipping, and the order of efficiency of exon skipping induced in patient cells mirrored that seen in normal cells (Table 1, and Fig. 1). Further, these PMOs generate the highest levels of exon skipping in patient cells over a range of concentrations (up to 200 nM) and, most important for potential therapeutic application, exert their activity at concentrations as low as 25 nM. The exon skipping activity of these PMOs is also persistent, with over 60% exon skipping for 21 days in culture for PMO-G. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMOs targeting the sequence +30+65 of exon 53 of the *DMD* gene were also shown to skip exon 53 correctly in vivo in the hDMD mouse. It should be noted that the levels of exon skipping in the hDMD mouse by each particular PMO was variable. This has been reported previously [12], and is likely to be due to the poor uptake into the non-dystrophic muscle of the hDMD mouse. However this does not compromise the importance of the finding that the PMOs tested here are able to elicit the targeted skipping of exon 53 in vivo. A summary of the relative efficacy of the different Type 1 PMOs tested over the different assays used is presented in Fig. 4a. The recommendation of PMO-G as a potential clinical trial reagent of choice for the targeted skipping of exon 53 of the *DMD* gene relative to the other Type 1 PMOs, is based primarily on it's more persistent longevity of action. Repeated administration of PMO will be required for prolonged antisense therapeutic action, and the prolonged action of PMO-G makes it an attractive choice with PMOs-A and -H providing viable alternatives if required. These RNA results were further confirmed by the detection of dystrophin protein in extracts from patient cells treated with these PMOs.

Although efficiency of exon skipping is perhaps the most important quality an AO can possess, it is not the only one. The potential for the PMOs targeting sequence +30+65 of exon 53 to have off-target effects could be considered to be negligible, since no completely homologous sequences were found on BLAST analysis (results not shown). There is a common single nucleotide polymorphism (SNP) seen on exon 53 of the *DMD* gene. PMOs -J, -K, -L and -M had this SNP (c7728C>T) in the last, fourth to last, seventh to last and second to last base of their target sites, respectively. There is the potential that this allelic mismatch could influence the binding and bioactivity of these PMOs. However, the more active PMOs (-A, -B, -G, -H and -I) all had their target sites away from the SNP, thus removing the possible effect of a mismatch weakening binding and bioactivity, allowing definitive comparisons between these PMOs to be made. The DMD patient (del 45–52) carried the normal (T) allele, hence the SNP would not affect the binding of the PMOs that anneal at this site. Additionally, there was no evidence that the PMOs produced cellular cytotoxicity (results not shown). This, together with the predicted stability of the PMO–target complexes [24], suggests these PMOs have potential as a clinical therapy.

We would therefore recommend that PMOs targeting sequence +30+65 of exon 53 of the *DMD* gene worthy of consideration for any upcoming clinical trial. In this study, sequence +30+65 was effectively targeted by PMOs-A, -B, -G and -H, resulting in exon 53 skipping. Since repeated delivery would be required for therapeutic action, the more persistent action of PMO-G may suggest this to be the PMO of choice for the targeted skipping of exon 53, and PMOs-A and -H providing viable alternatives if required.

Acknowledgements

The authors wish to thank members of the UK Muscular Dystrophy Exon skipping (MDEX) Consortium for helpful discussions, and the Muscular Dystrophy Campaign, Action Duchenne (formerly Parent Project UK), Duchenne Family Support Group, Muscular Dystrophy Ireland, NIH, Muscular Dystrophy Association USA, Clinigene EC Network of Excellence (LSHB-CT-2006018933) and participating patients for supporting this study. All work was carried out with informed consent and with the approval of the institutional ethics committee. The authors would also like to thank Prof. Ryszard Kole for his advice and careful reading of the paper. The authors also thank Janet Rose Christensen and Peter O'Hanley for the support of AVI Biopharma in the supply of PMO AOs. The work was funded by the Department of Health (UK). JEM was supported by a MRC collaborative career development fellowship in stem cell research and is currently funded by a Wellcome Trust University Award. CA was funded by the Muscular Dystrophy Campaign, Association Francais Contre les Myopathies, EU MYOAMP and is currently funded by the Duchenne Parent Project. The MRC Neuro-

muscular Centre support to the Biobank is also gratefully acknowledged.

References

[1] Hoffmann EP, Brown RH, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell 1987;51:919–28.

[2] Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988;2:90–5.

[3] Muntoni F, Wells D. Genetic treatments in muscular dystrophies. Curr Opin Neurol 2007;20:590–4.

[4] Mann CJ, Honeyman K, Cheng AJ, et al. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. Proc Natl Acad Sci USA 2001;98:42–7.

[5] Aartsma-Rus A, Janson AA, Kaman WE, et al. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. Hum Mol Genet 2003;12:907–14.

[6] van Deutekom JC, Bremmer-Bout M, Janson AA, et al. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum Mol Genet 2001;10:1547–54.

[7] Aartsma-Rus A, Janson AA, Kaman WE, et al. Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. Am J Hum Genet 2004;74:83–92.

[8] Aartsma-Rus A, Janson AA, van Ommen GJ, van Deutekom JCT. Antisense-induced exon skipping for duplications in Duchenne muscular dystrophy. BMC Med Genet 2007;8:43–51.

[9] Arechavala-Gomeza V, Graham IR, Popplewell LJ, et al. Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during pre-mRNA splicing in human muscle. Hum Gene Ther 2007;18:798–810.

[10] Lu QL, Mann CJ, Lou F, et al. Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nat Med 2003;9:1009–14.

[11] Graham IR, Hill VJ, Manoharan M, Inamati GB, Dickson G. Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligonucleotides (splicomers): target sequence optimisation using oligonucleotide arrays. J Gene Med 2004;6:1149–58.

[12] Bremmer-Bout M, Aartsma-Rus A, de Meijer EJ, et al. Targeted exon skipping in transgenic hDMD mice: a model for direct preclinical screening of human-specific antisense oligonucleotides. Mol Ther 2004;10:232–40.

[13] Jearawiriyapaisarn N, Moulton HM, Buckley B, et al. Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. Mol Ther 2008;16:1624–9.

[14] van Deutekom JC, Janson AA, Ginjaar IB, et al. Local antisense dystrophin restoration with antisense oligonucleotide PRO051. N Eng J Med 2007;357:2677–87.

[15] Kinali M, Arechavala-Gomeza V, Feng L, et al. Restoration of dystrophin expression in Duchenne muscular dystrophy: a single blind, placebo-controlled dose escalation study using morpholino oligomer AVI-4658. Lancet Neurol 2009;8:918–28.

[16] Arora V, Devi GR, Iversen PL. Neutrally charged phosphorodiamidate morpholino antisense oligomers: uptake, efficacy and pharmacokinetics. Curr Pharm Biotechnol 2004;5:431–9.

[17] Heemskerk HA, de Winter CL, de Kimpe SJ, et al. In vivo comparison of 2′-O-methyl-PS and morpholino antisense oligonucleotides for DMD exon skipping. J Gene Med 2009;11:257–66.

[18] Gebski BL, Mann CJ, Fletcher S, Wilton SD. Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. Hum Mol Genet 2003;12:1801–11.

[19] Alter J, Lou F, Rabinowitz A, et al. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat Med 2006;12:175–7.

[20] Malerba A, Thorogood FC, Dickson G, Graham IR. Dosing regimen has a significant impact on the efficiency of morpholino oligomer-induced exon skipping in mdx mice. Hum Gene Ther 2009;20:955–65.

[21] McClorey G, Fall AM, Moulton HM, et al. Induced dystrophin exon skipping in human muscle explants. Neuromuscul Disord 2006;16:583–90.

[22] McClorey G, Moulton HM, Iversen PL, Fletcher S, Wilton SD. Antisense oligonucleotide-induced exon skipping restores dystrophin expression *in vitro* in a canine model of DMD. Gene Ther 2006;13:1373–81.

[23] Aartsma-Rus A, Fokkema I, Verschuuren J, et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. Hum Mutat 2009;30:293–9.

[24] Popplewell LJ, Trollet C, Dickson G, Graham IR. Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene. Mol Ther 2009;17:554–61.

[25] Wilton SD, Fall AM, Harding PL, McClorey G, Coleman C, Fletcher S. Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript. Mol Ther 2007;15:1288–96.

[26] McCulley C, Adkin C, Fletcher S, Wilton SD, Morgan J, Wells DJ. Optimising the efficacy of morpholino antisense oligonucleotide induced exon-skipping in primary myoblasts using nucleofection, submitted for publication.

[27] 'tHoen PAC, de Meijer EJ, Boer JM, et al. Generation and characterization of transgenic mice with the full-length human DMD gene. J Biol Chem 2008;283:5899–907.

[28] Aartsma-Rus A, De Winter CL, Janson AAM, et al. Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites. Oligonucleotides 2005;15:284–97.

[29] Aartsma-Rus A, van Vliet L, Hirschi M, et al. Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. Mol Ther 2008;17:548–53.

[30] Harding PL, Fall AM, Honeyman K, Fletcher S, Wilton SD. The influence of antisense oligonucleotide length on dystrophin exon skipping. Mol Ther 2007;15:157–66.

[31] Aartsma-Rus A, Kaman WE, Bremmer-Bout M, et al. Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. Gene Ther 2004;11:1391–8.

Neuromuscular Disorders

Volume 20 Number 2 February 2010

Cited in: Current Contents/Life Sciences, Elsevier BIOBASE/Current Awareness in Biological Sciences, Index Medicus, MEDLINE, Neuroscience Citation Index, Reference Update, Research Alert, Science Citation Index, SciSearch

CONTENTS

0960-8966(201002)20:2;1-H

EXHIBIT AX

US 20100168212A1

(19) **United States**

(12) **Patent Application Publication**

(10) **Pub. No.: US 2010/0168212 A1**

(43) **Pub. Date: Jul. 1, 2010**

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(21) Appl. No.: **12/556,626**

(22) Filed: **Sep. 10, 2009**

Related U.S. Application Data

(60) Provisional application No. 61/096,073, filed on Sep. 11, 2008, provisional application No. 61/164,978, filed on Mar. 31, 2009.

Publication Classification

(51) **Int. Cl.**

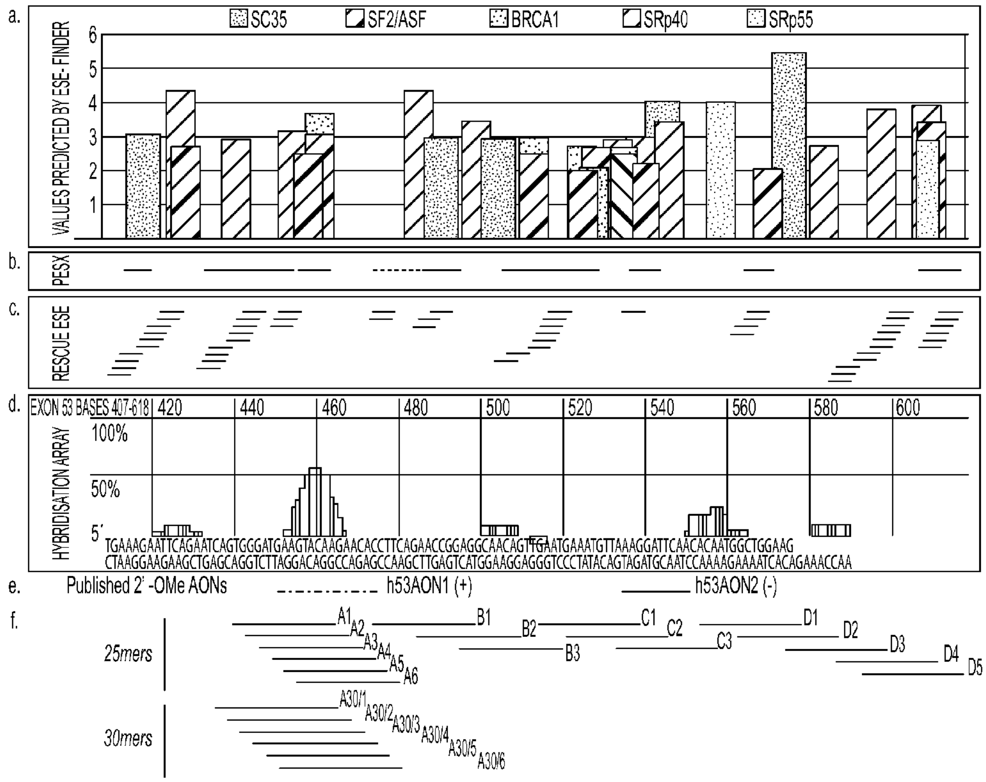
A61K 31/7088	(2006.01)
C07H 21/04	(2006.01)
C12N 15/63	(2006.01)

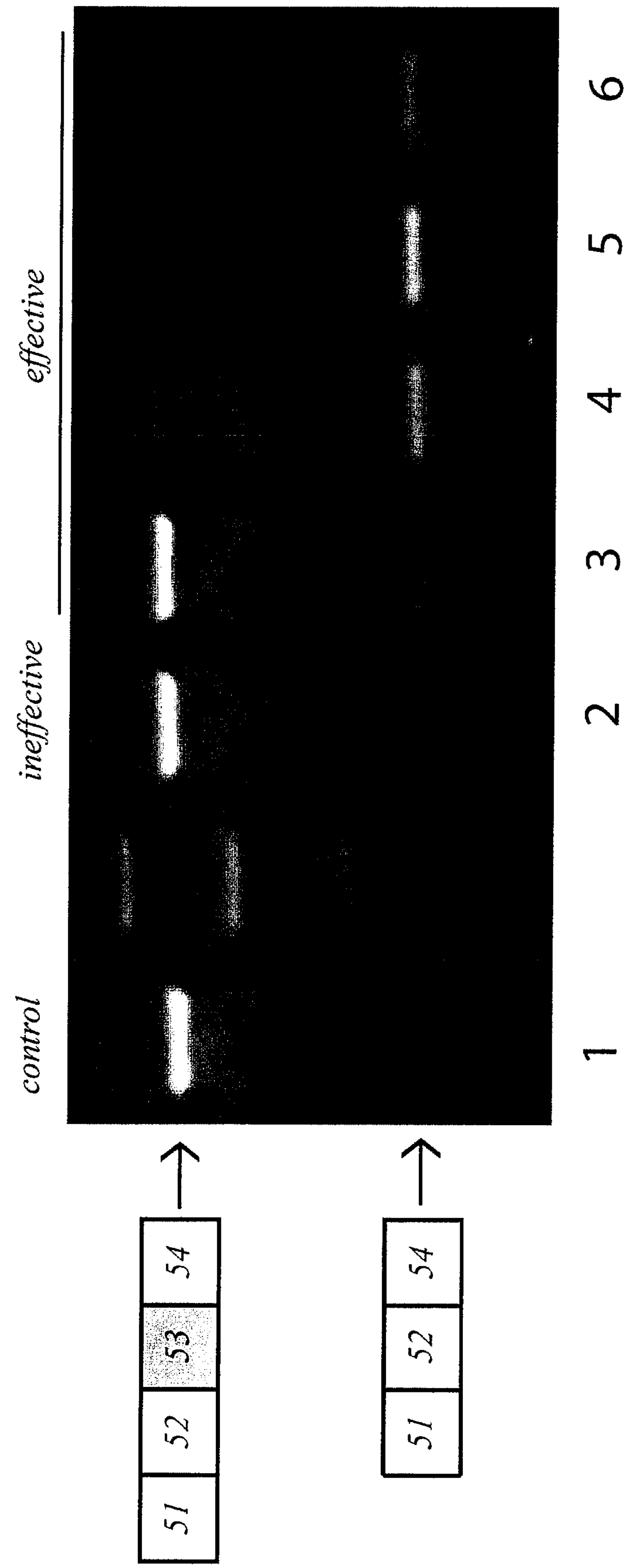
(52) **U.S. Cl.** .... **514/44 R**; 536/23.1; 435/320.1

(57) **ABSTRACT**

Molecules are provided for inducing or facilitating exon skipping in forming spliced mRNA products from pre-mRNA molecules in cells. The molecules may be provided directly as oligonucleotides or expression products of vectors that are administered to a subject. High rates of skipping can be achieved. High rates of skipping reduce the severity of a disease like Duchene Muscular Dystrophy so that the disease is more like Becker Muscular Dystrophy. This is a severe reduction in symptom severity and mortality.

FIG. 1


a.
SC35
SF2/ASF
BRCA1
SRp40
SRp55
VALUES PREDICTED BY ESE- FINDER
b.
PESX
c.
RESCUE ESE
d.
EXON 53 BASES 407-618
HYBRIDISATION ARRAY
100%
50%
5'
TGAAAGAATTCAGAATCAGTGGGATGAAGTACAAGAACACCTTCAGAACCGGAGGCAACAGTTGAATGAAATGTTAAAGGATTCAACACAATGGCTGGAAG
CTAAGGAAGAAGCTGAGCAGGTCTTAGGACAGGCCAGAGCCAAGCTTGAGTCATGGAAGGAGGGTCCCTATACAGTAGATGCAATCCAAAAGAAAATCACAGAAACCAA
e.
Published 2'-OMe AONs
h53AON1 (+)
h53AON2 (-)
f.
25mers
30mers



2

Figure 2

Figure 3

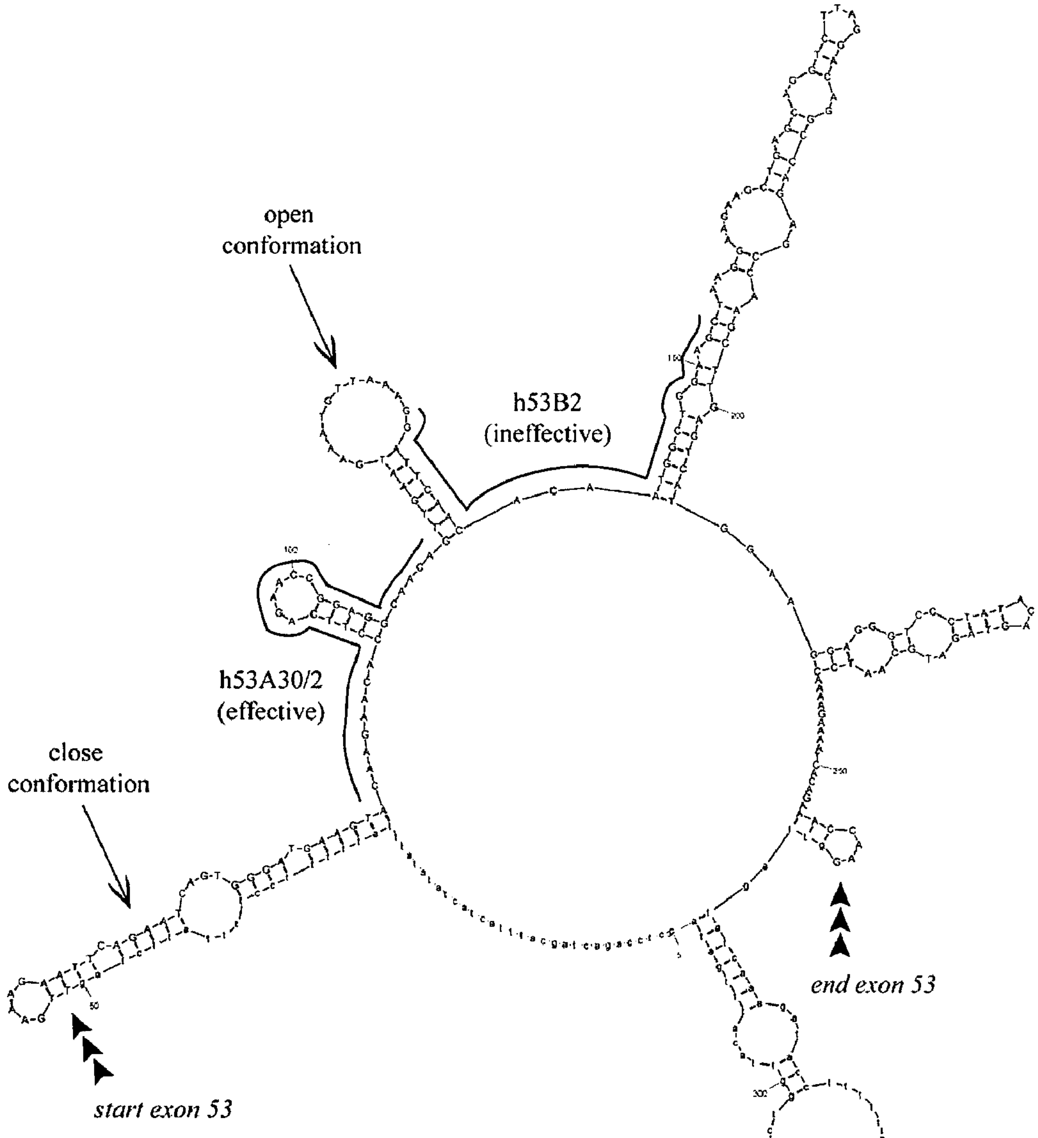
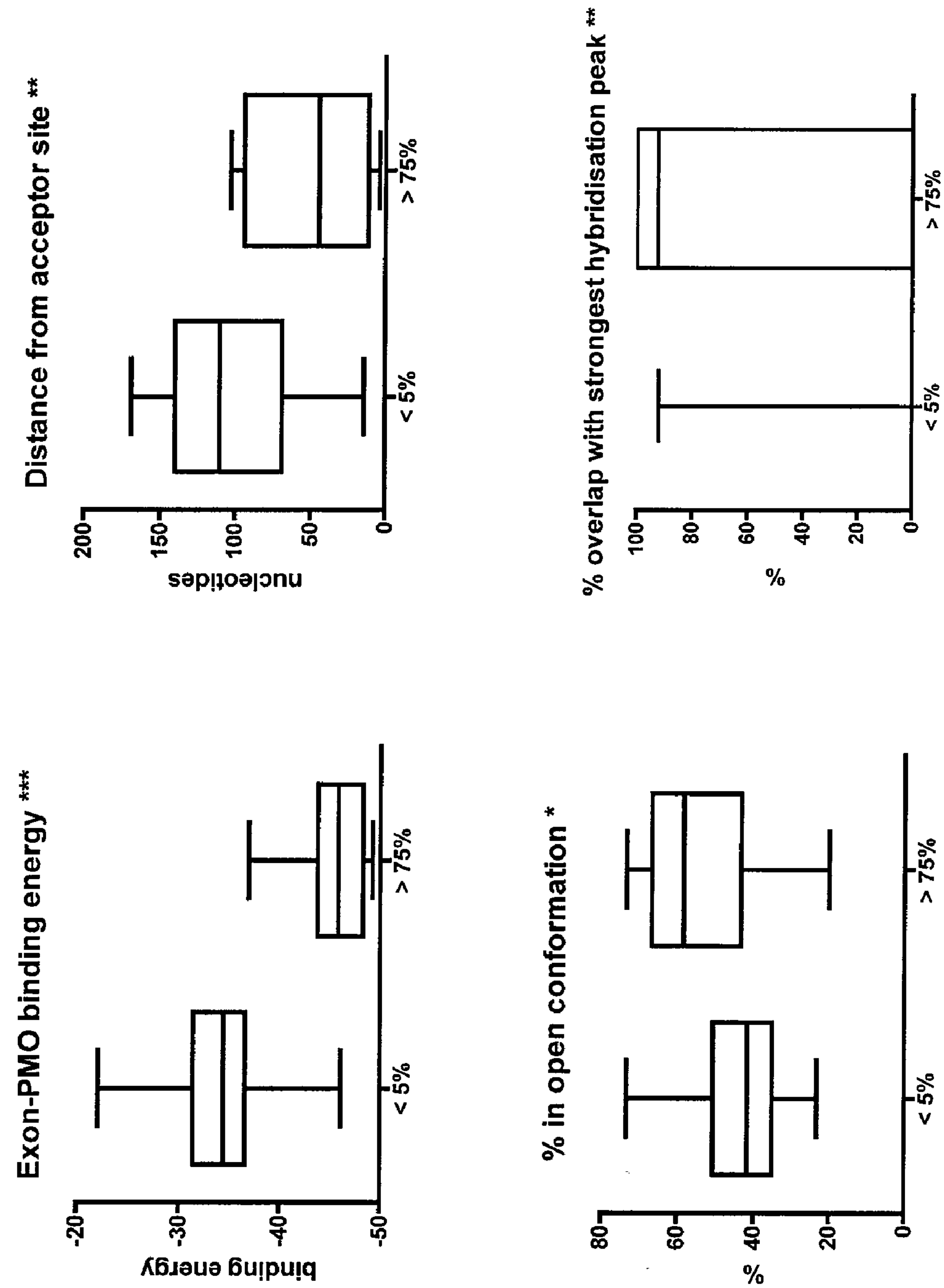
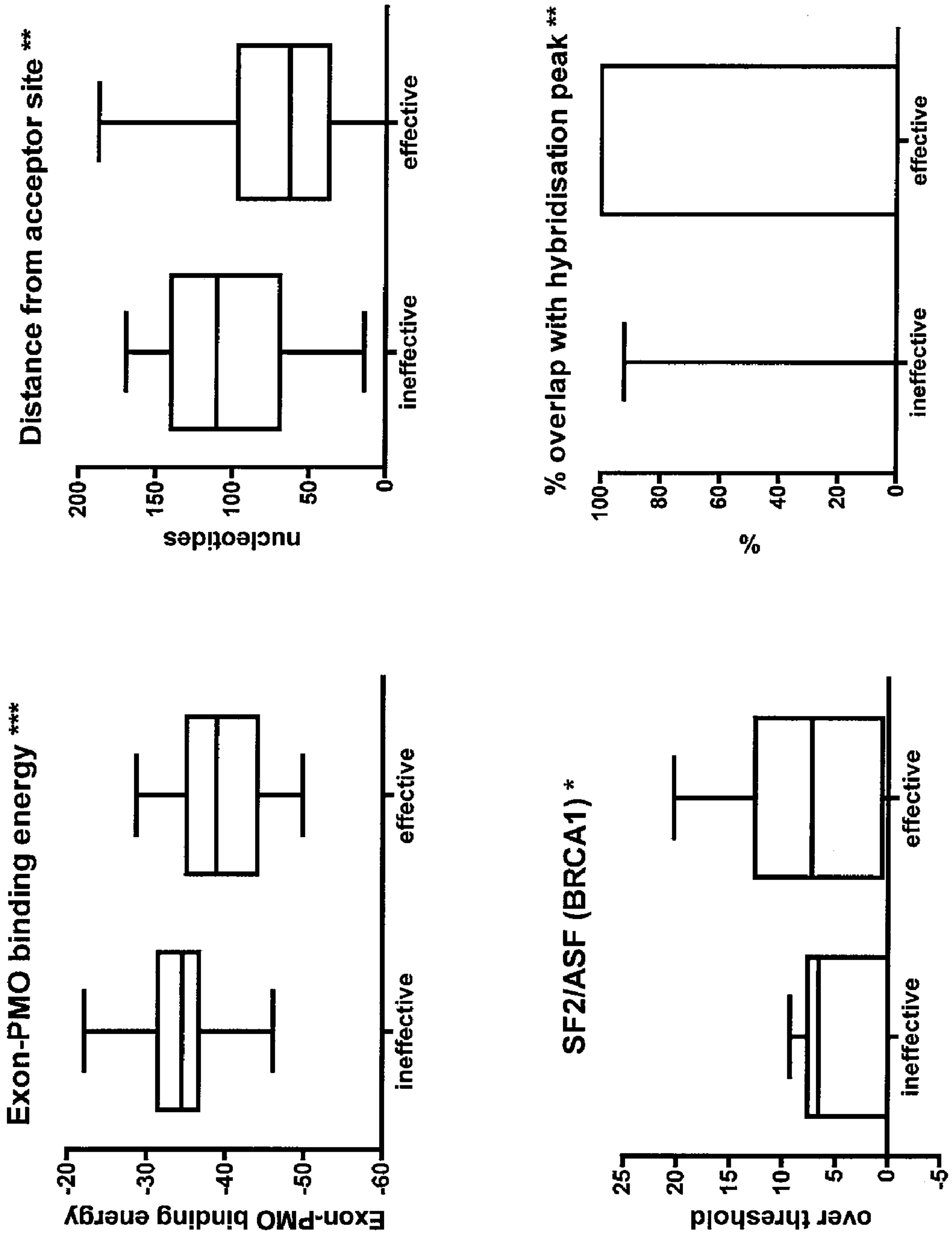

open conformation
h53B2 (ineffective)
h53A30/2 (effective)
close conformation
end exon 53
start exon 53

Figure 4


Exon-PMO binding energy ***
binding energy
-20
-30
-40
-50
< 5%
> 75%
Distance from acceptor site **
nucleotides
200
150
100
50
0
< 5%
> 75%
% in open conformation *
%
80
60
40
20
0
< 5%
> 75%
% overlap with strongest hybridisation peak **
%
100
80
60
40
20
0
< 5%
> 75%

A-D

Figure 5A-D


Exon-PMO binding energy ***
Exon-PMO binding energy
-20
-30
-40
-50
-60
ineffective
effective
Distance from acceptor site **
nucleotides
200
150
100
50
0
ineffective
effective
SF2/ASF (BRCA1) *
over threshold
25
20
15
10
5
0
-5
ineffective
effective
% overlap with hybridisation peak **
%
100
80
60
40
20
0
ineffective
effective

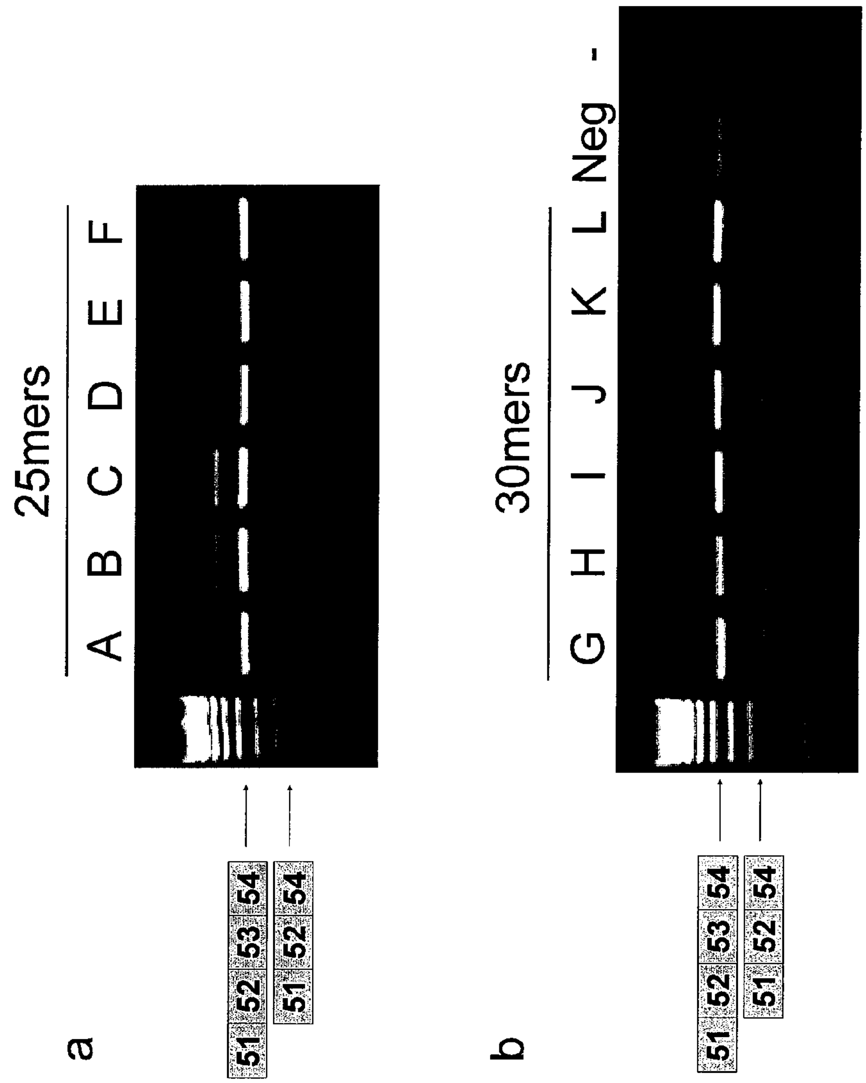
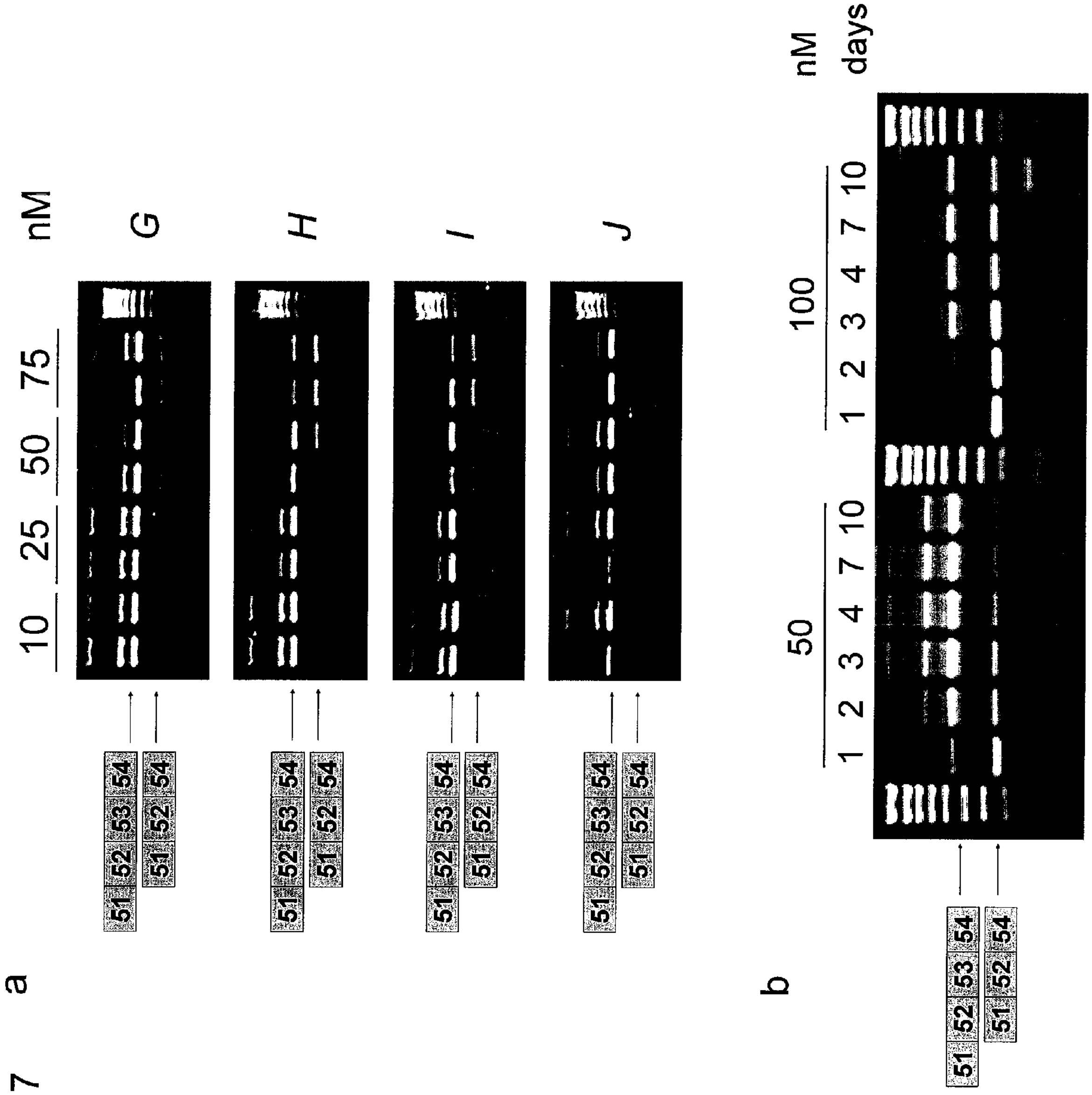

a
25mers
A B C D E F
51 52 53 54
51 52 54
b
30mers
G H I J K L Neg -
51 52 53 54
51 52 54

Figure 6

Figure 7


a
nM
10
25
50
75
G
H
I
J
51 52 53 54
51 52 54
b
nM
days
50
100
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1 2 3 4 7 10

Figure 8

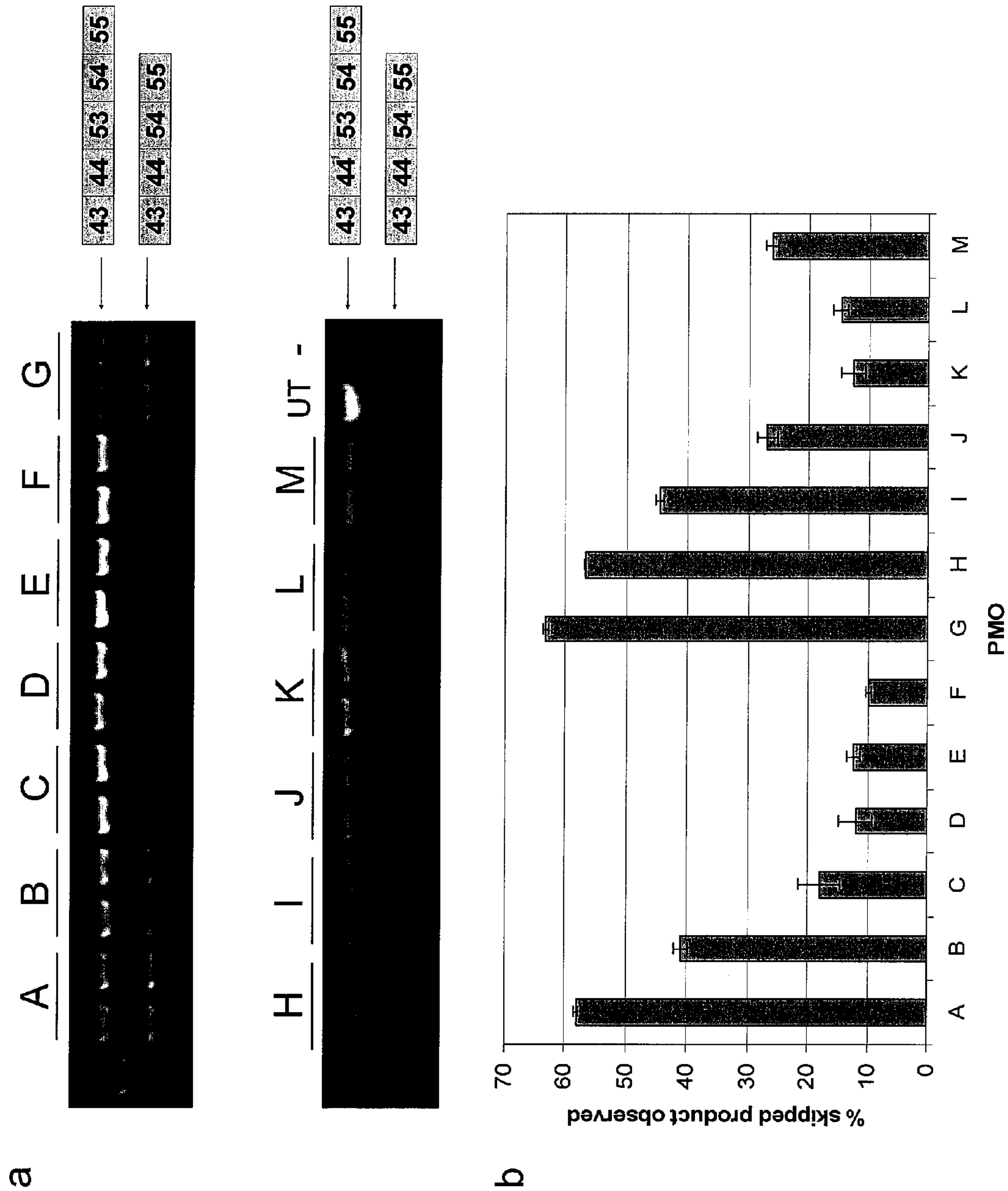

a
A
B
C
D
E
F
G
H
I
J
K
L
M
UT -
43 44 53 54 55
43 44 54 55
b
% skipped product observed
0
10
20
30
40
50
60
70
PMO

Figure 9

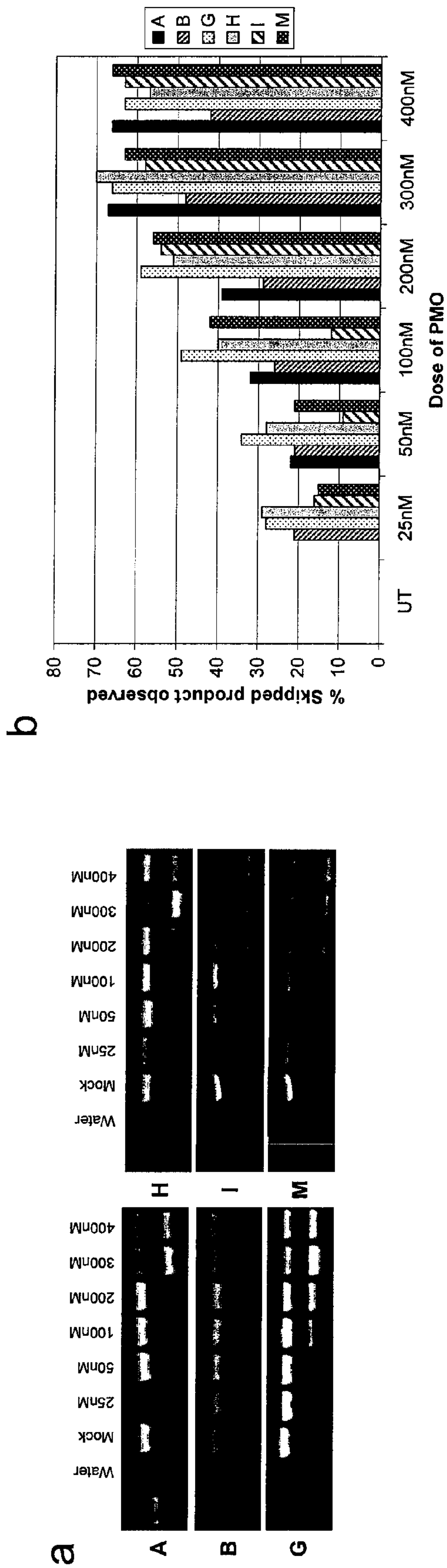
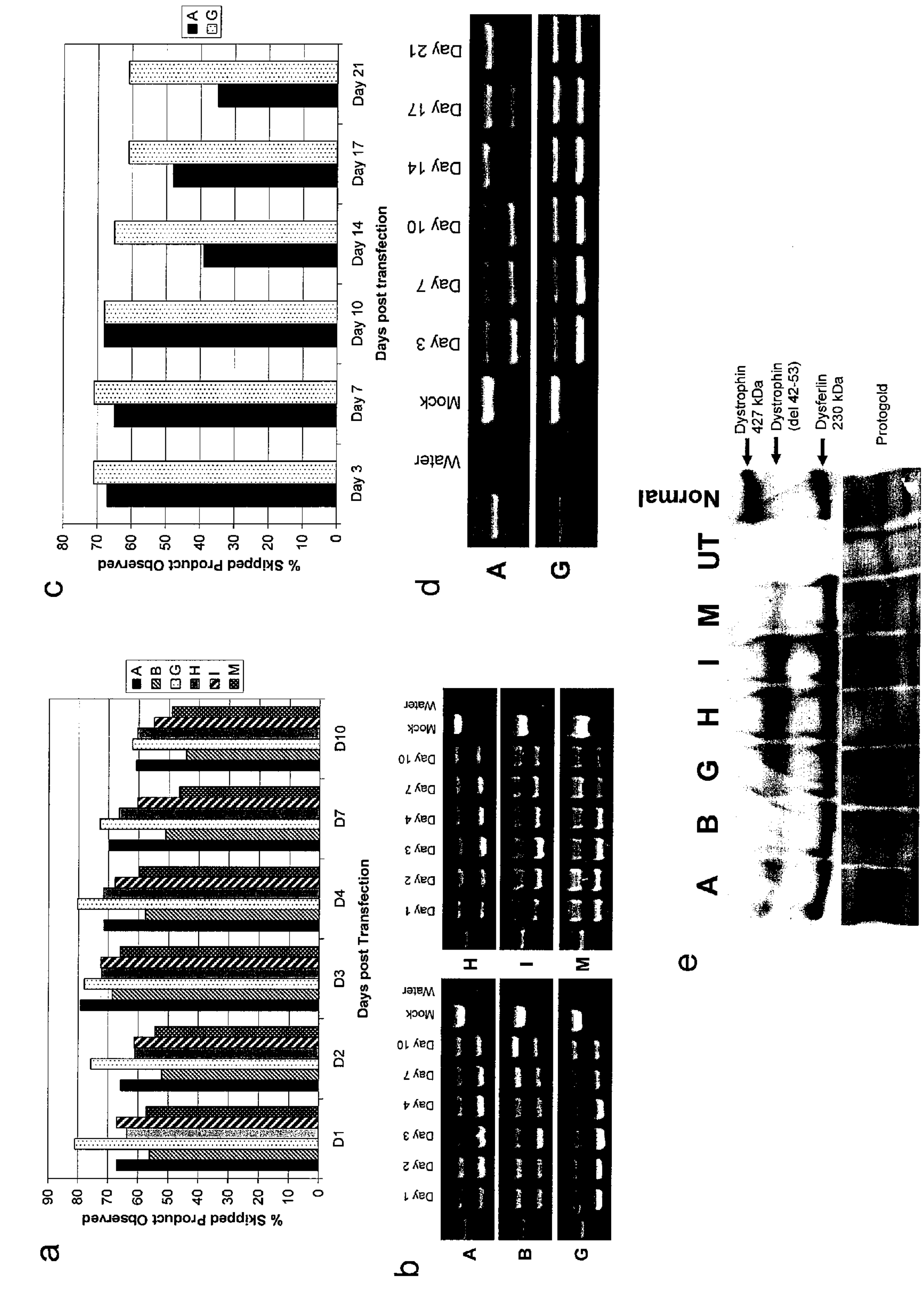

a
A
B
G
H
I
M
Water
Mock
25nM
50nM
100nM
200nM
300nM
400nM
b
% Skipped product observed
Dose of PMO
UT
0
10
20
30
40
50
60
70
80

Figure 10


a
% Skipped Product Observed
Days post Transfection
D1
D2
D3
D4
D7
D10
b
c
Days post transfection
Day 3
Day 7
Day 10
Day 14
Day 17
Day 21
d
Water
Mock
e
Normal
Dystrophin 427 kDa
Dystrophin (del 42-53)
Dysferlin 230 kDa
Protogold

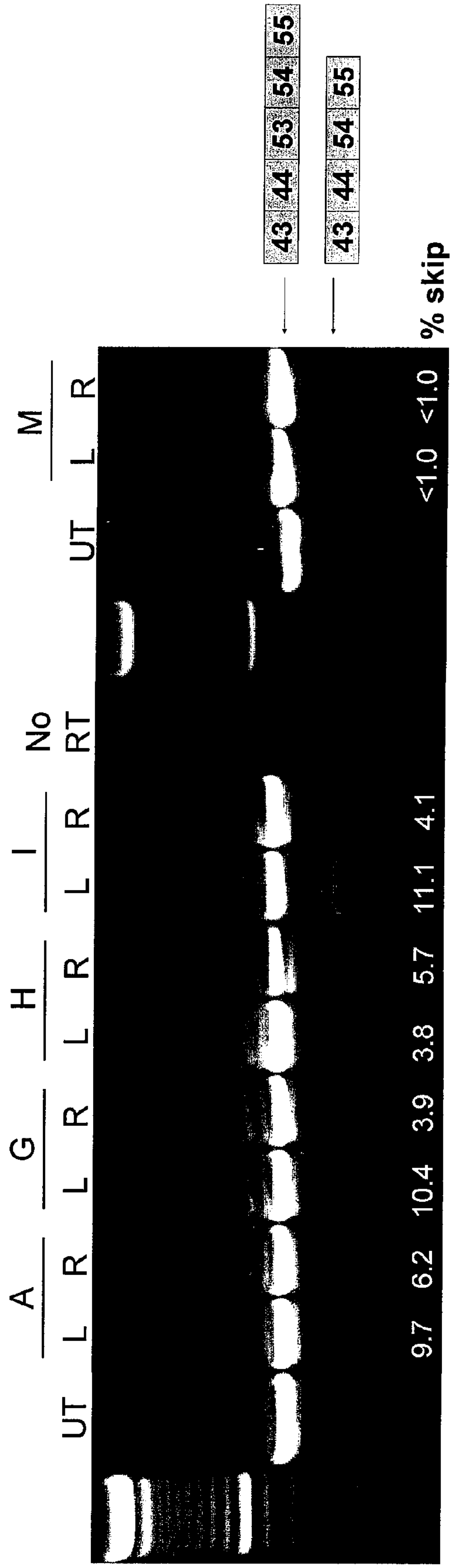

UT
A
L R
G
L R
H
L R
I
L R
No RT
UT
M
L R
43 44 53 54 55
43 44 54 55
% skip
9.7 6.2 10.4 3.9 3.8 5.7 11.1 4.1 <1.0 <1.0

Figure 11

OLIGOMERS

TECHNICAL FIELD OF THE INVENTION

[0001] The present invention relates to molecules which are capable of causing exon skipping and, in particular, relates to molecules which are capable of causing exon skipping in the dystrophin gene.

BACKGROUND OF THE INVENTION

[0002] Duchenne muscular dystrophy (DMD) is a severe X-linked muscle wasting disease, affecting 1:3500 boys. Prognosis is poor: loss of mobility by the age of 12, compromised respiratory and cardiac function by late teens, and probable death by the age of 30. The disease is caused by mutations within the large dystrophin gene, such that the reading frame is disrupted leading to lack of dystrophin protein expression and breakdown of muscle fibre integrity [1]. The dystrophin gene is large, with 79 exons. The most common DMD mutation is genomic deletion of one or more exons, generally centred around hotspots involving exons 44 to 55 and the 5' end of the gene [2]. Mutations of the dystrophin gene that preserve the reading frame result in the milder, non-life threatening Becker muscular dystrophy (BMD).

[0003] Exon skipping induced by antisense oligoribonucleotides (AOs), generally based on an RNA backbone, is a future hope as a therapy for DMD in which the effects of mutations in the dystrophin gene can be modulated through a process of targeted exon skipping during the splicing process. The splicing process is directed by complex multi-particle machinery that brings adjacent exon-intron junctions in pre-mRNA into close proximity and performs cleavage of phosphodiester bonds at the ends of the introns with their subsequent reformation between exons that are to be spliced together. This complex and highly precise process is mediated by sequence motifs in the pre-mRNA that are relatively short semi-conserved RNA segments to which bind the various nuclear splicing factors that are then involved in the splicing reactions. By changing the way the splicing machinery reads or recognises the motifs involved in pre-mRNA processing, it is possible to create differentially spliced mRNA molecules.

[0004] It has now been recognised that the majority of human genes are alternatively spliced during normal gene expression, although the mechanisms involved have not been identified. Using antisense oligonucleotides, it has been shown that errors and deficiencies in a coded mRNA could be bypassed or removed from the mature gene transcripts. Indeed, by skipping out-of-frame mutations of the dystrophin gene, the reading frame can be restored and a truncated, yet functional, Becker-like dystrophin protein is expressed. Studies in human cells in vitro [3, 4] and in animal models of the disease in vivo [5-9] have proven the principle of exon skipping as a potential therapy for DMD (reviewed in [10]). Initial clinical trials using two different AO chemistries (phosphorodiamidate morpholino oligomer (PMO) and phosphorothioate-linked 2'-β-methyl RNA (2'OMePS)) [11] have recently been performed, with encouraging results. Indisputably impressive restoration of dystrophin expression in the TA muscle of four DMD patients injected with a 2'OMePS AO to exon 51 has been reported by van Deutekom et al. [11].

[0005] However, it should be noted that, relative to 2'OMePS AOs, PMOs have been shown to produce more consistent and sustained exon skipping in the mdx mouse model of DMD [12-14; A. Malerba et al, manuscript submitted], in human muscle explants [15], and in dystrophic canine cells in vitro [16]. Most importantly, PMOs have excellent safety profiles from clinical and pre-clinical data [17].

[0006] The first step to a clinical trial is the choice of the optimal AO target site for skipping of those dystrophin exons most commonly deleted in DMD. In depth analysis of arrays of 2'OMePS AOs have been reported [18, 19], and relationships between skipping bioactivity and AO variables examined.

[0007] One problem associated with the prior art is that the antisense oligonucleotides of the prior art do not produce efficient exon skipping. This means that a certain amount of mRNA produced in the splicing process will contain the out-of-frame mutation which leads to protein expression associated with DMD rather than expression of the truncated, yet functional, Becker-like dystrophin protein associated with mRNA in which certain exons have been skipped.

[0008] Another problem associated with the prior art is that antisense oligonucleotides have not been developed to all of the exons in the dystrophin gene in which mutations occur in DMD.

[0009] An aim of the present invention is to provide molecules which cause efficient exon skipping in selected exons of the dystrophin gene, thus being suitable for use in ameliorating the effects of DMD.

SUMMARY OF THE INVENTION

[0010] The present invention relates to molecules which can bind to pre-mRNA produced from the dystrophin gene and cause a high degree of exon skipping in a particular exon. These molecules can be administered therapeutically.

[0011] The present invention provides a molecule for ameliorating DMD, the molecule comprising at least a 25 base length from a base sequence selected from:

```
                                           (SEQ ID NO: 1)
a)   XGA AAA CGC CGC CAX XXC XCA ACA GAX CXG;

                                           (SEQ ID NO: 2)
b)   CAX AAX GAA AAC GCC GCC AXX XCX CAA CAG;

                                           (SEQ ID NO: 3)
c)   XGX XCA GCX XCX GXX AGC CAC XGA XXA AAX;

                                           (SEQ ID NO: 4)
d)   CAG XXX GCC GCX GCC CAA XGC CAX CCX GGA;

                                           (SEQ ID NO: 5)
e)   XXG CCG CXG CCC AAX GCC AXC CXG GAG XXC;

                                           (SEQ ID NO: 6)
f)   XGC XGC XCX XXX CCA GGX XCA AGX GGG AXA;

                                           (SEQ ID NO: 7)
g)   CXX XXA GXX GCX GCX CXX XXC CAG GXX CAA;

                                           (SEQ ID NO: 8)
h)   CXX XXC XXX XAG XXG CXG CXC XXX XCC AGG;

                                           (SEQ ID NO: 9)
i)   XXA GXX GCX GCX CXX XXC CAG GXX CAA GXG;

                                          (SEQ ID NO: 10)
j)   CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;

                                          (SEQ ID NO: 11)
k)   CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;
```

-continued

or

(SEQ ID NO: 12)
l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's base sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in an exon of the dystrophin gene.

[0012] The exon of the dystrophin gene is selected from exons 44, 45, 46 or 53. More specifically, the molecule that causes skipping in exon 44 comprises at least a 25 base length from a base sequence selected from:

(SEQ ID NO: 1)
a) XGA AAA CGC CGC CAX XXC XCA ACA GAX CXG;

(SEQ ID NO: 2)
b) CAX AAX GAA AAC GCC GCC AXX XCX CAA CAG;
or

(SEQ ID NO: 3)
c) XGX XCA GCX XCX GXX AGC CAC XGA XXA AAX,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 44 of the dystrophin gene.

[0013] The molecule that causes skipping in exon 45 comprises at least a 25 base length from a base sequence selected from:

(SEQ ID NO: 4)
d) CAG XXX GCC GCX GCC CAA XGC CAX CCX GGA;
or

(SEQ ID NO: 5)
e) XXG CCG CXG CCC AAX GCC AXC CXG GAG XXC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 45 of the dystrophin gene.

[0014] The molecule that causes skipping in exon 46 comprises at least a 25 base length from a base sequence selected from:

(SEQ ID NO: 6)
f) XGC XGC XCX XXX CCA GGX XCA AGX GGG AXA;

(SEQ ID NO: 7)
g) CXX XXA GXX GCX GCX CXX XXC CAG GXX CAA;

(SEQ ID NO: 8)
h) CXX XXC XXX XAG XXG CXG CXC XXX XCC AGG;
or

(SEQ ID NO: 9)
i) XXA GXX GCX GCX CXX XXC CAG GXX CAA GXG,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 46 of the dystrophin gene.

[0015] The molecule that causes skipping in exon 53 comprises at least a 25 base length from a base sequence selected from:

(SEQ ID NO: 10)
j) CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;

(SEQ ID NO: 11)
k) CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;
or

(SEQ ID NO: 12)
l) XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,

wherein the molecule's sequence can vary from the above sequence at up to two base positions, and wherein the molecule can bind to a target site to cause exon skipping in exon 53 of the dystrophin gene.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. **1** shows a scheme summarizing the tools used in the design of PMOs to exon 53. (a) Results of ESEfinder analysis, showing the location and values above threshold for SF2/ASF, SF2/ASF (BRCA1), SC35, SRp40 and SRp55, shown as grey and black bars, as indicated in the legend above. (b) Output of PESX analysis, showing the location of exonic splicing enhancers as solid lines, and exonic splicing silencer as a dashed line. (c) Rescue ESE analysis for exon 53, showing predicted ESEs by lines, and where they overlap, by a ladder of lines. (d) AccessMapper analysis of in vitro hybridization. Synthetic pre-mRNA containing exon 53 and surrounding introns was subjected to a hybridization screen against a random hexamer oligonucleotide array, as described in Materials and Methods. Areas of hybridization, suggestive of areas of open conformation, are indicated by peaks on the graph. (e) The position of the target sites of two 2'OMePS AOs studied previously [18] are shown for comparison. (f) The location of the target sites for all the 25mer and 30mer PMOs to exon 53 used in this study are indicated by lines, and numbered according to the scheme used in Table 1, except for exclusion of the prefix "h53";

[0017] FIG. **2** shows a comparison of active (effective) and inactive (ineffective) PMOs. RT-PCR analysis of mRNA from normal human skeletal muscle cells treated with PMOs to exon 53 demonstrates a wide variation in the efficiency of exon skipping. Over 75% exon skipping is seen with h53A30/2 (lane 5) and h53A30/3 (lane 6). h53A30/1 (lane 4) produced around 50% skipping, while the 25-mer h53A1 (lane 3) produced just over 10% skipping. In contrast, h53C1 (lane 2) was completely inactive. Lane 1 contains a negative control in which cells were treated with lipofectin but no PMO.

[0018] FIG. **3** shows an Mfold secondary structure prediction for exon 53 of the human dystrophin gene. MFOLD analysis [25] was performed using exon 53 plus 50 nt of the upstream and downstream introns, and with a maximum base-pairing distance of 100 nt. The intron and exon boundaries are indicated, as are the positions of the target sites of the bioactive PMO h53A30/2 (87.2% skip) and an inactive PMO (h53B2). Examples of open and closed RNA secondary structure are arrowed.

[0019] FIG. **4** shows boxplots of parameters significant to strong PMO bioactivity. Comparisons were made between inactive PMOs and those inducing skipping at levels in excess of 75%. Boxplots are shown for parameters which are signifi-

cant on a Mann-Whitney rank sum test: PMO to target binding energy, distance of the target site from the splice acceptor site, the percentage overlap with areas of open conformation, as predicted by MFOLD software, and the percentage overlap of the target site with the strongest area accessible to binding, as revealed by hexamer hybridization array analysis. Degrees of significance are indicated by asterisks. *: p<0.05; **: p<0.01; ***: p<0.001.

[0020] FIG. **5** shows boxplots of parameters significantly different between bioactive (effective) and inactive (ineffective) PMOs. Comparisons were made between PMOs determined as bioactive (those that induced skipping at greater than 5%) and those that were not. Boxplots are shown for parameters which are significant from a Mann-Whitney rank sum test: PMO to target binding energy, distance of the target site from the splice acceptor site, the score over threshold for a predicted binding site for the SR protein SF2/ASF, and the percentage overlap of the target site with the strongest area accessible to binding, as revealed by hexamer hybridization array analysis. Degrees of significance are indicated by asterisks. *: p<0.05; **: p<0.01; ***: p<0.001.

[0021] FIG. **6** shows a comparison of bioactivity of PMOs targeted to exon 53 in normal hSkMCs. Myoblasts were transfected with each of the 25mer (panel a) and 30mer (panel b) PMOs indicated at 500 nM using lipofectin (1:4). RNA was harvested after 24 hours and subjected to nested RT-PCR and products visualised by agarose gel electrophoresis.

[0022] FIG. **7** shows low dose efficacy and timecourse of skipping of the most bioactive PMOs in normal hSkMCs. (a) hSkMC myoblasts were transfected with the PMOs indicated over a concentration range of 25 nM to 100 nM using lipofectin (1:4). RNA was harvested after 24 hours and subjected to nested RT-PCR, and products visualised by agarose gel electrophoresis. (b) hSkMC myoblasts were transfected with 100 nM and 500 nM concentrations of PMO-G (+30+59) using lipofectin. RNA was harvested at the timepoints indicated following transfection and subjected to nested RT-PCR, and products visualised by agarose gel electrophoresis. Skipped (248 bp) and unskipped (460 bp) products are shown schematically.

[0023] FIG. **8** shows blind comparison of 13 PMO oligonucleotide sequences to skip human exon 53. Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45-52 were transfected at 300 nM in duplicate with each of the PMOs by nucleofection. RNA was harvested 3 days following transfection, and amplified by nested RT-PCR. (a) Bars indicate the percentage of exon skipping achieved for each PMO, derived from Image J analysis of the electropherogram of the agarose gel (b). Skipped (477 bp) and unskipped (689 bp) products are shown schematically.

[0024] FIG. **9** shows the dose-response of the six best-performing PMOs. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45-52 were transfected with the six best-performing PMOs by nucleofection, at doses ranging from 25 nM to 400 nM. RT-PCR products derived from RNA isolated from cells 3 days post-transfection were separated by agarose gel electrophoresis. (b) The percentage of exon skipping observed is expressed for each concentration of each PMO as a comparison of the percentage OD of skipped and unskipped band, as measured using Image J.

[0025] FIG. **10** shows persistence of dystrophin expression in DMD cells following PMO treatment. (a) Myoblasts derived from a DMD patient carrying a deletion of dystrophin exons 45-52 were transfected by nucleofection at 300 nM with each of the six best-performing PMOs, and were cultured for 1 to 10 days before extracting RNA. The percentage of exon skipping was compared using the percentage OD of skipped and unskipped bands, measured using Image J analysis of the agarose gel of the nested RT-PCR products shown in (b). The experiment was repeated, but this time using the two best-performing PMOs from the previous analysis, and continuing the cultures for 21 days post-transfection (c and d). (e) Western blot analysis was performed on total protein extracts from del 45-52 DMD cells 7 days after transfection with the six best PMOs (300 nM). Blots were probed with antibodies to dystrophin, to dysferlin as a muscle-specific loading control, and protogold for total protein loading control. CHQ5B myoblasts, after 7 days of differentiation were used as a positive control for dystrophin protein (normal).

[0026] FIG. **11** shows a comparison of most active PMOs in hDMD mice. PMOs were injected in a blind experiment into the gastrocnemius muscle of hDMD mice. RT-PCR analysis of RNA harvested from isolated muscle (L=left, R=right) was performed and products visualised by agarose gel electrophoresis. Quantification of PCR products was performed using a DNA LabChip.

DETAILED DESCRIPTION OF THE INVENTION

[0027] Without being restricted to any particular theory, it is thought by the inventors that the binding of the molecules to the dystrophin pre-mRNA interacts with or interferes with the binding of SR proteins to the exon of interest. SR proteins are involved in the slicing process of adjacent exons. Therefore, it is thought that interacting or interfering with the binding of the SR proteins interferes with the splicing machinery resulting in exon skipping.

[0028] The base "X" in the above base sequences is defined as being thymine (T) or uracil (U). The presence of either base in the sequence will still allow the molecule to bind to the pre-mRNA of the dystrophin gene as it is a complementary sequence. Therefore, the presence of either base in the molecule will cause exon skipping. The base sequence of the molecule may contain all thymines, all uracils or a combination of the two. One factor that can determine whether X is T or U is the chemistry used to produce the molecule. For example, if the molecule is a phosphorodiamidate morpholino oligonucleotide (PMO), X will be T as this base is used when producing PMOs. Alternatively, if the molecule is a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS), X will be U as this base is used when producing 2'OMePSs. Preferably, the base "X" is only thymine (T).

[0029] The advantage provided by the molecule is that it causes a high level of exon skipping. Preferably, the molecule causes an exon skipping rate of at least 50%, more preferably, at least 60%, even more preferably, at least 70%, more preferably still, at least 76%, more preferably, at least 80%, even more preferably, at least 85%, more preferably still, at least 90%, and most preferably, at least 95%.

[0030] The molecule can be any type of molecule as long as it has the selected base sequence and can bind to a target site of the dystrophin pre-mRNA to cause exon skipping. For example, the molecule can be an oligodeoxyribonucleotide, an oligoribonucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO) or a phosphorothioate-linked 2'-O-methyl oligonucleotide (2'OMePS). Preferably, the oligonucleotide is a PMO. The advantage of a PMO is that it has excellent safety profiles and appears to have longer lasting

effects in vivo compared to 2'OMePS oligonucleotides. Preferably, the molecule is isolated so that it is free from other compounds or contaminants.

[0031] The base sequence of the molecule can vary from the selected sequence at up to two base positions. If the base sequence does vary at two positions, the molecule will still be able to bind to the dystrophin pre-mRNA to cause exon skipping. Preferably, the base sequence of the molecule varies from the selected sequence at one base position and, more preferably, the base sequence does not vary from the selected sequence. The less that the base sequence of the molecule varies from the selected sequence, the more efficiently it binds to the specific exon region in order to cause exon skipping.

[0032] The molecule is at least 25 bases in length. Preferably, the molecule is at least 28 bases in length. Preferably, the molecule is no more than 35 bases in length and, more preferably, no more than 32 bases in length. Preferably, the molecule is between 25 and 35 bases in length, more preferably, the molecule is between 28 and 32 bases in length, even more preferably, the molecule is between 29 and 31 bases in length, and most preferably, the molecule is 30 bases in length. It has been found that a molecule which is 30 bases in length causes efficient exon skipping. If the molecule is longer than 35 bases in length, the specificity of the binding to the specific exon region is reduced. If the molecule is less than 25 bases in length, the exon skipping efficiency is reduced.

[0033] The molecule may be conjugated to or complexed with various entities. For example, the molecule may be conjugated to or complexed with a targeting protein in order to target the molecule to muscle tissue. Alternatively, the molecule may be complexed with or conjugated to a drug or another compound for treating DMD. If the molecule is conjugated to an entity, it may be conjugated directly or via a linker. In one embodiment, a plurality of molecules directed to exon skipping in different exons may be conjugated to or complexed with a single entity. Alternatively, a plurality of molecules directed to exon skipping in the same exon may be conjugated to or complexed with a single entity. For example, an arginine-rich cell penetrating peptide (CPP) can be conjugated to or complexed with the molecule. In particular, (R-Ahx-R)(4)AhxB can be used, where Ahx is 6-aminohexanoic acid and B is beta-alanine [35], or alternatively (RXRRBR)2XB can be used [36]. These entities have been complexed to known dystrophin exon-skipping molecules which have shown sustained skipping of dystrophin exons in vitro and in vivo.

[0034] In another aspect, the present invention provides a vector for ameliorating DMD, the vector encoding a molecule of the invention, wherein expression of the vector in a human cell causes the molecule to be expressed. For example, it is possible to express antisense sequences in the form of a gene, which can thus be delivered on a vector. One way to do this would be to modify the sequence of a U7 snRNA gene to include an antisense sequence according to the invention. The U7 gene, complete with its own promoter sequences, can be delivered on an adeno-associated virus (AAV) vector, to induce bodywide exon skipping. Similar methods to achieve exon skipping, by using a vector encoding a molecule of the invention, would be apparent to one skilled in the art.

[0035] The present invention also provides a pharmaceutical composition for ameliorating DMD, the composition comprising a molecule as described above or a vector as described above and any pharmaceutically acceptable carrier, adjuvant or vehicle. Pharmaceutical compositions of this invention comprise any molecule of the present invention, and pharmaceutically acceptable salts, esters, salts of such esters, or any other compound which, upon administration to a human, is capable of providing (directly or indirectly) the biologically active molecule thereof, with any pharmaceutically acceptable carrier, adjuvant or vehicle. Pharmaceutically acceptable carriers, adjuvants and vehicles that may be used in the pharmaceutical compositions of this invention include, but are not limited to, ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts or electrolytes, such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, polyethylene glycol, sodium carboxymethylcellulose, polyacrylates, waxes, polyethylene-polyoxypropylene-block polymers, polyethylene glycol and wool fat.

[0036] The pharmaceutical compositions of this invention may be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally, intradermally or via an implanted reservoir. Oral administration or administration by injection is preferred. The pharmaceutical compositions of this invention may contain any conventional non-toxic pharmaceutically-acceptable carriers, adjuvants or vehicles. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrathecal, intralesional and intracranial injection or infusion techniques. Preferably, the route of administration is by injection, more preferably, the route of administration is intramuscular, intravenous or subcutaneous injection and most preferably, the route of administration is intravenous or subcutaneous injection.

[0037] The pharmaceutical compositions may be in the form of a sterile injectable preparation, for example, as a sterile injectable aqueous or oleaginous suspension. This suspension may be formulated according to techniques known in the art using suitable dispersing or wetting agents (such as, for example, Tween 80) and suspending agents. The sterile injectable preparation may also be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are mannitol, water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil may be employed including synthetic mono- or diglycerides. Fatty acids, such as oleic acid and its glyceride derivatives are useful in the preparation of injectables, as are natural pharmaceutically acceptable oils, such as olive oil or castor oil, especially in their polyoxyethylated versions. These oil solutions or suspensions may also contain a long-chain alcohol diluent, dispersant or similar alcohol.

[0038] The pharmaceutical compositions of this invention may be orally administered in any orally acceptable dosage form including, but not limited to, capsules, tablets, and aqueous suspensions and solutions. In the case of tablets for oral use, carriers which are commonly used include lactose and corn starch. Lubricating agents, such as magnesium stearate, are also typically added. For oral administration in a capsule

form, useful diluents include lactose and dried corn starch. When aqueous suspensions are administered orally, the active ingredient is combined with emulsifying and suspending agents. If desired, certain sweetening and/or flavouring and/or colouring agents may be added.

[0039] The pharmaceutical compositions of this invention may also be administered in the form of suppositories for rectal administration. These compositions can be prepared by mixing a compound of this invention with a suitable non-irritating excipient which is solid at room temperature but liquid at the rectal temperature and therefore will melt in the rectum to release the active components. Such materials include, but are not limited to, cocoa butter, beeswax and polyethylene glycols.

[0040] Topical administration of the pharmaceutical compositions of this invention is especially useful when the desired treatment involves areas or organs readily accessible by topical application. For application topically to the skin, the pharmaceutical composition should be formulated with a suitable ointment containing the active components suspended or dissolved in a carrier. Carriers for topical administration of the compounds of this invention include, but are not limited to, mineral oil, liquid petroleum, white petroleum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, the pharmaceutical composition can be formulated with a suitable lotion or cream containing the active compound suspended or dissolved in a carrier. Suitable carriers include, but are not limited to, mineral oil, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. The pharmaceutical compositions of this invention may also be topically applied to the lower intestinal tract by rectal suppository formulation or in a suitable enema formulation. Topically-transdermal patches are also included in this invention.

[0041] The pharmaceutical compositions of this invention may be administered by nasal aerosol or inhalation. Such compositions are prepared according to techniques well-known in the art of pharmaceutical formulation and may be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other solubilizing or dispersing agents known in the art.

[0042] In one embodiment, the pharmaceutical composition may comprise a plurality of molecules of the invention, each molecule directed to exon skipping in a different exon. Alternatively, the pharmaceutical composition may comprise a plurality of molecules of the invention, each molecule directed to exon skipping in the same exon.

[0043] In another embodiment, the pharmaceutical composition may comprise a plurality of vectors of the invention, each vector encoding a molecule directed to exon skipping in a different exon. Alternatively, the pharmaceutical composition may comprise a plurality of vectors of the invention, each vector encoding a molecule directed to exon skipping in the same exon.

[0044] In yet another embodiment, the pharmaceutical composition may comprise a molecule and a vector, wherein the molecule and the molecule encoded by the vector are directed to exon skipping in the same or different exons.

[0045] The present invention also provides a molecule of the invention for use in therapy.

[0046] Further, the present invention provides a molecule of the invention for use in the amelioration of DMD.

[0047] The molecules of the present invention cause exon skipping in the dystrophin pre-mRNA. This causes a truncated but functional dystrophin protein to be expressed which results in a syndrome similar to Becker muscular dystrophy (BMD). Therefore, the symptoms of DMD will not be completely treated but will be ameliorated so that they are potentially no longer life threatening.

[0048] The present invention also provides a method of ameliorating DMD in a human patient, the method comprising administering a therapeutically effective amount of the molecule of the invention to the patient.

[0049] The particular molecule that is administered to the patient will depend on the location of the mutation or mutations present in the dystrophin gene of the patient. The majority of patients have deletions of one or more exons of the dystrophin gene. For example, if a patient is missing exon 44, the process of joining exon 43 to exon 45 will destroy the protein, thus causing DMD. If exon 45 is skipped using a molecule of the invention, the joining of exon 43 to exon 46 will restore the protein. Similarly, a patient with a deletion of exon 45 can be treated with a molecule to skip either exon 44 or exon 46. Further, a patient with a deletion of exons 45 to 52 inclusive (a large portion of the gene), would respond to skipping of exon 53.

[0050] In another aspect, the invention provides a kit for the amelioration of DMD in a patient, the kit comprising a molecule of the invention and instructions for its use. In one embodiment, the kit may contain a plurality of molecules for use in causing exon skipping in the same exon or a plurality of exons.

EXAMPLES

Example 1

[0051] Here, the first detailed study of the role that AO target site variables have on the efficacy of PMOs to induce skipping is reported. The results reported here should have an impact on the initial planning and design of AOs for future potential clinical trials.

Materials and Methods

Hybridization Analyses

[0052] Templates for the production of synthetic pre-mRNAs for exons 44, 45, 46, 51, and 53 of the human dystrophin gene (DMD gene) were generated by PCR amplification from genomic clones of the exons, together with approximately 500 nt of upstream and downstream introns. PCR primers incorporated T7 RNA polymerase promoter sequences, such that pre-mRNAs could be produced by in vitro transcription. Pre-mRNAs were then subjected to a hybridization screen against a spotted array of all 4096 possible hexanucleotide sequences (Access Array 4000; Nyrion Ltd, Edinburgh UK). Binding of the pre-mRNA to specific spots on the array was detected by reverse transcriptase-mediated incorporation of biotinylated nucleotides by primer extension, followed by fluorescent labelling. Scanning of the arrays followed by software analysis enabled sequences within the exons that were accessible to binding to the hexamer array to be identified. Using a hybridization assay, binding accessibility of

each exons were analysed and hybridization peak identified by AccessMapper software (Nyrion Ltd) (see FIG. **1***d*).

AO Design

[0053] Overlapping AOs were designed to exons 44, 45, 46, 51, and 53 of the human DMD gene using the following information: putative SR protein binding domains as predicted by ESEfinder [20, 21], Rescue ESE [24] and PESX [22, 23] analyses of exon sequence; sequences accessible to binding as determined by hybridization analyses (Nyrion); previously published work [18, 19].

[0054] All AOs were synthesized as phosphorodiamidate morpholino oligos (PMOs) by Gene Tools LLC (Philomath Oreg., USA). To facilitate transfection of these uncharged oligonucleotides into cultured cells, the PMOs were hybridized to phosphorothioate-capped oligodeoxynucleotide leashes, as described by Gebski et al., [12], and stored at 4° C.

[0055] The sequences of some of these PMOs were as follows:

```
                                          (SEQ ID NO: 13)
H44A30/1 -
TGA AAA CGC CGC CAT TTC TCA ACA GAT CTG;

                                          (SEQ ID NO: 14)
H44A30/2 -
CAT AAT GAA AAC GCC GCC ATT TCT CAA CAG;

                                          (SEQ ID NO: 15)
H44AB30/2 -
TGT TCA GCT TCT GTT AGC CAC TGA TTA AAT;

                                          (SEQ ID NO: 16)
H45A30/2 -
CAG TTT GCC GCT GCC CAA TGC CAT CCT GGA;

                                          (SEQ ID NO: 17)
H45A30/1 -
TTG CCG CTG CCC AAT GCC ATC CTG GAG TTC;

                                          (SEQ ID NO: 18)
H46A30/2 -
TGC TGC TCT TTT CCA GGT TCA AGT GGG ATA;

                                          (SEQ ID NO: 19)
H46A30/4 -
CTT TTA GTT GCT GCT CTT TTC CAG GTT CAA;

                                          (SEQ ID NO: 20)
H46A30/5 -
CTT TTC TTT TAG TTG CTG CTC TTT TCC AGG;

                                          (SEQ ID NO: 21)
H46A30/3 -
TTA GTT GCT GCT CTT TTC CAG GTT CAA GTG;

                                          (SEQ ID NO: 22)
H53A30/2 -
CTG TTG CCT CCG GTT CTG AAG GTG TTC TTG;

                                          (SEQ ID NO: 23)
H53A30/3 -
CAA CTG TTG CCT CCG GTT CTG AAG GTG TTC;

                                          (SEQ ID NO: 24)
H53A30/1 -
TTG CCT CCG GTT CTG AAG GTG TTC TTG TAC.
```

Cell Culture and AO Transfection

[0056] Normal human primary skeletal muscle cells (TCS Cellworks, Buckingham, UK) were seeded in 6-well plates coated with 0.1 mg/ml ECM Gel (Sigma-Aldrich, Poole, UK), and grown in supplemented muscle cell growth medium (Promocell, Heidelberg, Germany). Cultures were switched to supplemented muscle cell differentiation medium (Promocell) when myoblasts fused to form visible myotubes (elongated cells containing multiple nuclei and myofibrils). Transfection of PMOs was then performed using the transfection reagent Lipofectin (Invitrogen, Paisley, UK) at a ratio of 4 µl of Lipofectin per µg of PMO (with a range of PMO concentrations tested from 50 to 500 nM, equivalent to approximately 0.5 to 5 µg) for 4 hrs, according to the manufacturer's instructions. All transfections were performed in triplicate in at least two different experiments.

RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction Analysis

[0057] Typically 24 h after transfection, RNA was extracted from the cells using the QIAshredder/RNeasy system (Qiagen, Crawley, UK) and ~200 ng RNA subjected to RT-PCR with DMD exon-specific primers using the GeneScript kit (Genesys, Camberley, UK). From this 20 cycle reaction, an aliquot was used as a template for a second nested PCR consisting of 25 cycles. PCR products were analysed on 1.5% agarose gels in Tris-borate/EDTA buffer. Skipping efficiencies were determined by quantification of the PCR products by densitometry using GeneTools software (Syngene, Cambridge, UK).

Statistical Analysis

[0058] The non-parametric Mann-Whitney rank sum test was used to identify whether parameters for effective PMOs were significantly different to those for ineffective PMOs. Where data was calculated to fit a normal distribution, the more powerful two-tailed Student's t-test was performed instead. Correlations were generated using the Spearman rank-order test. To determine the strength of the combined significant parameters/design tools to design effective PMOs, linear discriminant analysis was used [34], with the Ida function from the MASS package, using "effective" or "ineffective" as the two prior probabilities. The Ida function produces posterior probabilities for the two classes (effective and ineffective) for each PMO by leave-one-out classification.

Results

PMO Design and Analysis of Bioactivity

[0059] A unique set of 66 PMOs has been designed to target exons 44, 45, 46, 51, and 53 of the human gene for dystrophin. The design process for exon 53 is depicted in FIG. **1**, and has also been performed for the other four exons (data not shown). The exon sequence was analysed for the presence of exonic splicing enhancers (ESE) and exonic splicing suppressors or silencers (ESS) and the outputs aligned for the three available algorithms, ESEfinder (FIG. **1***a*) [20, 21], PESX (FIG. **1***b*) [22, 23], and Rescue ESE (FIG. **1***c*) [24]. Hybridization array analysis was also performed for each exon in vitro, as described in Materials and Methods. The peaks shown in FIG. **1***d* indicate areas of the exon that are in a conformation able to hybridize to the array, and which may consequently prove more accessible to antisense AOs. The coincidence of ESEs, as predicted by two or more algorithms, and hybridization peaks determined experimentally, was used to design arrays of 25mer and, subsequently, 30mer PMOs,

the positions of which are shown in FIG. **1***f*. The binding sites for 2'OMePS AOs described previously [18] are shown for comparison (FIG. **1***e*).

[0060] Each PMO was tested in primary cultures of human skeletal muscle, in triplicate, in at least two experiments, and over a range of concentrations from 50 nM to 500 nM. Their bioactivity was determined by RT-PCR analysis, which showed a wide variation in the level of exon skipping induced (FIG. **2**, and data not shown), ranging from 0% for h53C1 (FIG. **1***f* and FIG. **2**, lane 2) to 80% for h53A30/3 (FIG. **1***f* and FIG. **2**, lane 6). Sequencing of the PCR products verified accurate skipping of the targeted exon (data not shown). The activity of each PMO at the stated optimal concentration is summarized in Table 1. Bioactivity is expressed as a percentage of the skipped amplicon relative to total PCR product, as assessed by densitometry. Specific, consistent and sustained exon skipping was evident for 44 of the 66 PMOs tested.

In Silico Analysis of PMOs

[0061] We then performed a retrospective in silico analysis of the characteristics of all 66 PMOs tested in this study, with respect to PMO length, the distance of the PMO target site from the splice donor and acceptor sites, PMO-to-target binding energy and PMO-to-PMO binding energy, as calculated using RNAstructure2.2 software for the equivalent RNA-RNA interaction, and percentage GC content of the PMO, the results of which are summarized in Table 1. Also shown in Table 1 is the percentage overlap of each PMO target site with sequences shown to be accessible to binding, as determined experimentally by the hexamer hybridization array analysis. The relationship of PMO target site and RNA secondary structure was also examined using the program MFOLD [25] (FIG. **3** and data not shown), with the percentage overlap of PMO target site with sequence predicted to be in open conformation by MFOLD analysis given in Table 1. ESEfinder and SSF (http://www.umd.be/SSF/) software analysis of exon sequences revealed the positions of putative SR protein binding motifs (SF2/ASF (by two algorithms), SC35, SRp40, SRp55, Tra2β and 9G8). The highest score over threshold for each SR protein is given for each PMO in the columns on the right of Table 1. Also shown is the degree of overlap of each PMO target site with the ESE and ESS regions predicted by Rescue ESE and PESX.

Statistical Analysis of Design Parameters in Relation to PMO Bioactivity

[0062] For this statistical analysis, bioactive PMOs are considered to be those which produce over 5% skipping, while those that produce less than 5% skipping are considered inactive. For each of the parameters listed in Table 1, comparison was made between bioactive and inactive PMOs using the non-parametric Mann-Whitney rank sum test, or, when it was statistically valid to do so, the parametric Student's t-test (two-tailed). The significant parameters are listed in Table 2. Considering the data as a whole, the variable which showed the highest significance to PMO bioactivity was the binding energy of the PMO to the exon (p=0.001); the most bioactive exons are predicted to bind better to their target sites. Those PMOs that overlap with peaks identified by the experimental hybridization array analysis are not significantly more active than those that do not (p=0.056), but when only the strongest peak for each exon is considered, this parameter becomes highly significant (p=0.003). Distance of the PMO target site to the splice acceptor site of the exon was also highly significant (p=0.004), with PMOs whose target site were closer to the acceptor site being more active. PMOs whose target sites showed coincidence with binding motifs for the SR protein SF2/ASF (as defined by the BRCA1 algorithm of Smith et al. [21]) produced significantly greater skipping (p=0.026). PMO length is also a significant parameter (p=0.017), with longer PMOs being more effective at inducing skipping. Boxplots of the significant variables identified here are shown in FIG. **5**. None of the other variables considered in this study were shown to have any significance to AO bioactivity.

[0063] To ascertain which parameters/design tools are the most powerful, we also used the Mann-Whitney rank sum test to compare the most active PMOs (i.e. those that induce greater than 75% skipping of the target exon) to those that were inactive (i.e. those that produce less than 5% skipping). Boxplots of the significant variables for this comparison are shown in FIG. **4**. There is strong significance of overlap of the PMO target site with the strongest hybridization peak for each exon (p=0.002); more of the most bioactive PMOs had their target sites coincident with sequences accessible to binding than those that were inactive. This is reinforced by the observation that the target sites of PMOs that produced over 75% skipping significantly overlapped more RNA that was in open conformation, relative to inactive PMOs (p=0.025). Stronger binding between the PMO and its target exon, PMO length, and proximity of the target to the acceptor site are also significant parameters when comparing the most and least effective reagents. Spearman's rank order correlation was used to establish potential relationships between design parameters and skipping bioactivity using the entire set of PMOs. This shows a strong correlation between skipping bioactivity and PMO-target binding energy (r_s=−0.618, p=0), percentage open conformation (r_s=0.275, p=0.0259), PMO length (r_s=0.545, p=0), distance from the splice acceptor site (r_s=−0.421, p=0), percentage overlap with the strongest hybridization peak (r_s=0.46, p=0), and overlap with an ESS sequence, as predicted by PESX (r_s=0.261, p=0.0348).

Linear Discriminant Analysis

[0064] This analysis was performed on all possible combinations of length, overlap with the SF2/ASF (BRCA1) motif, percentage overlap with areas of open conformation, percentage overlap with hybridization peak and PMO-target binding energy, i.e. PMO parameters and design tools that showed significance or borderline significance. Using length, SF2/ASF (BRCA1) motif and hybridization peak data, nine of the inactive PMOs were classified as bioactive and four bioactive PMOs were classified as inactive (Table 3). These four misclassified PMOs were 25mers to exon 46, three of which have borderline bioactivity, i.e. produce just 10% skipping, while the fourth produces about 20% skipping. Taken overall, this equates to 80% of the PMOs being predicted correctly when assessed according to their length, SF2/ASF (BRCA1) overlap and hybridization peak overlap. This would suggest that these parameters have the potential to be effective design tools, with four out of every five PMOs designed to have these three properties likely to be bioactive. In line with this, there was a distinct trend for PMOs being correctly assigned as bioactive with increased skipping bioactivity (see Table 3). Indeed, the PMOs with greatest bioactivity were all 30mers (10/10), bound to their target with a high binding energy of below −43.0 kD (9/10), overlapped by over 50% with areas of

open conformation (7/10), overlapped with SF2/ASF (BRCA1) peak (8/10), and overlapped with a hybridization peak (7/10).

Discussion

[0065] Clinical studies using AOs to skip exon 51 to correct DMD deletions are progressing well [11; F. Muntoni, Principal Investigator of MDEX Consortium, personal communication]. However since the mutations that cause DMD are so diverse, skipping of exon 51 would have the potential to treat just 24.6% of DMD patients on the Leiden DMD database [26]. It is therefore imperative that pre-clinical optimization of AO target sequence and chemistry is continually studied and improved. This study has examined the significance of design parameters for PMO-induced skipping of exons 44, 45, 46, 51, and 53, which would have the potential to treat, respectively, 11.5%, 15.8%, 8.4%, 24.6% and 13.5% of DMD patients in the Leiden database [26; A. Aartsma-Rus, personal communication].

[0066] Specific skipping was observed for the five DMD exons studied here, with two-thirds of the PMOs tested being bioactive. This proportion of bioactive AOs within a cohort has been reported previously [18, 19], but we have induced high-level (i.e. greater than 75%) skipping in four of the five exons tested, some of which are achievable at relatively low doses of oligomer. The exception is exon 51, published previously [4], achieving a maximal skipping of 26%. The work of Wilton et al [19] demonstrated that only exons 51 and 53 can be skipped with high efficiency (>30% by their definition), and that exons 44, 45 and 46 are less "skippable" (less than 30% skipping). Furthermore, Aartsma-Rus et al [18] showed oligomers capable of high-level skipping (greater than a mere 25%) for only exons 44, 46 and 51.

[0067] We provide here direct evidence that AO bioactivity shows a significant association with accessibility of its target site to binding. This is the first study to assess sequences practically within the pre-mRNA that are accessible to binding and then use them as an aid to AO design. The data we show underline the value of the hybridization analysis in determining what are likely to be the most bioactive oligomers (i.e. those that produce greater than 75% skipping). As an example, if we look at the data for oligomers developed for exon 45 [18], we see that there is only one moderately effective (5-25%) reagent for this otherwise unskippable exon. This oligomer is the only one of the six tested that overlaps with the strongest peak in our hybridization analysis. The partial nature of this overlap, combined with the short length of the oligomer, is likely to contribute to its relative weakness compared to the PMOs we have developed here. In general, the 2'OMePS AOs displaying the highest bioactivity in the work of Aartsma-Rus et al. [18] and Wilton et al. [19], show some degree of overlap with the hybridization peaks that we have defined here for exons 45, 46 and 53.

[0068] Ease of skipping of certain DMD exons has been seen elsewhere [18] and may be related to other factors affecting splicing, including strength of splice donor and acceptor sites and branchpoint, and the size of upstream and downstream introns, which may affect the order in which exons are spliced together. There is the potential of using a cocktail of AOs to induce greater skipping of the more difficult to skip exons [27, 28].

[0069] Accessibility of the AO to its target site depends directly on the secondary structure of the pre-mRNA, which has a major role in determining AO bioactivity in cells. A study in which the structure around an AO target site was changed revealed that AOs were unable to invade very stable stem-loop structures and their antisense activity was inhibited, but generally showed good activity when impeded by little local structure [29]. Overlap of PMO target sites with open conformations in the folded RNA showed a weak association with PMO bioactivity, which was more obvious when only the stronger PMOs were considered in the statistical analysis. It is also possible that there is selective pressure for SR binding sites to be located preferentially on these open secondary structures. The presumption is that binding of bioactive PMOs to their target sites sterically block the binding of important factors involved in RNA processing, resulting in exon skipping.

[0070] One of the PMO parameters with high significance was length; 30mer PMOs were far superior to their 25mer counterparts. The influence of 2'OMePS AO length on bioactivity has been reported elsewhere [30] and such an observation for PMO-induced skipping of exon 51 has been reported previously by us [4]. The more persistent action of longer PMOs would have important cost and dose implications in the choice of AO for clinical trials. Longer AOs are likely to sterically block more of the regions that interact with the splicing machinery, but in general terms, the energy of binding of the longer PMO to its target would be increased, which we showed to be the most significant parameter in AO design. The strong significance of the binding energy of PMO-target complexes (i.e., free energy of AO-target compared to free energy of the target) and PMO length to bioactivity suggests that PMO bioactivity depends on stability of the PMO-target complex, and implies that bioactive PMOs act by interference of target RNA folding. Computational analysis revealed that the thermodynamics of binding of active PMOs to their target site had a dramatic effect on the secondary folded structure of the RNA (data not shown). It is likely that these changes in secondary structure would have a profound effect on the binding of SR proteins to the RNA, thereby disrupting splicing, and exon skipping would ensue.

[0071] Overlap of a PMO target site with a binding site motif for the SR protein SF2/ASF (BRCA1), as predicted by ESEfinder, showed a significant association to PMO bioactivity. This partly confirms the work of Aartsma-Rus et al. [18], who observed marginally significantly higher ESEfinder values for SF2/ASF and SC35 motifs for effective AOs when compared to inactive AOs. SC35 and SF2/ASF motifs are the two most abundant proteins assessed by ESEfinder. The reason why we do not see any significance of overlap with SC35 motif to PMO bioactivity may be due to the difference in AO chemistry used, and the number of AOs assessed. However Aartsma-Rus et al. [18] did note that not every bioactive AO has a high value for any of the SR protein binding motifs, and some inactive AOs have high values. The apparent weakness and unreliability of SR protein binding motifs as design tools for AOs may be a reflection of the lack of precision of the predictive software used. Overlap of PMO target site with exonic splicing silencers appears to show a correlation with bioactivity in Spearman's rank order test analysis. Such a correlation would be counter-intuitive and the true significance questionable. Again the strength of the predictive software used may be in doubt. It should be noted that the software programmes used predict SR binding motifs on the linear exon sequence. The availability of these predicted motifs to bind SR proteins, or for binding PMOs to disrupt the binding of these proteins, is directly related to the

folding of the pre-mRNA. The discrepancy in the relative significance of secondary RNA structure and SR protein binding motifs may be due to active PMOs disrupting SR protein binding, not sterically but indirectly, by altering the secondary pre-mRNA structure. A very recent study has shown the importance of co-transcriptional pre-mRNA folding in determining the accessibility of AO target sites and their effective bioactivity, and showed a direct correlation between AO bioactivity and potential interaction with pre-mRNA [31].

[0072] It has been previously reported that ESE sites located within 70 nucleotides of a splice site are more active than ESE sites beyond this distance [32]. Our results partially support this; PMOs with their target site closer to the splice acceptor site are significantly more bioactive. However distance of the PMO target site to the splice donor site showed no statistical significance to bioactivity. This bias has been previously reported for the analyses of 2'OMePS AOs [18, 19], and may be related to the demonstration, by Patzel et al. [33], of the importance of an unstructured 5' end of RNA in the initiation of hybridization of oligonucleotide binding. This would suggest that targeting any significant parameters located in the 5' part of an exon may increase the probability of designing a bioactive AO.

[0073] In conclusion, our findings show that no single design tool is likely to be sufficient in isolation to allow the design of a bioactive AO, and empirical analysis is still required. However this study has highlighted the potential of using a combination of significant PMO parameters/design tools as a powerful aid in the design of bioactive PMOs. Linear discriminant analysis revealed that using the parameters of PMO length, overlap with SF2/ASF (BRCA1) motif and hexamer array hybridization data in combination would have an 80% chance of designing a bioactive PMO, which is an exciting and suprising finding, and should be exploited in further studies.

TABLE 1

Table 1: Table summarizing the characteristics of PMOs used

PMO	Targeted exon	Optimol conc.	% Skip[a]	Length	% GC	Exon-PMO binding energy	PMO-PMO binding energy	% open[b]	Ends in open loops[b]	Distance from donor	acceptor
h53B1	53	500	0	25	28	−22.1	−12.1	53.3	1	119	68
h53C1	53	500	0	25	48	−32.4	−9.8	46.7	2	79	108
h53C2	53	500	0	25	56	−31.3	−12.7	33.3	1	72	115
h53C3	53	500	0	25	60	−34.6	−13.7	26.7	1	60	127
h53D1	53	500	0	25	52	−34.1	−13.4	30	1	39	148
h45A30/4	45	500	0	30	43	−35.2	−7.5	40	1	43	93
h45A30/6	45	500	0	30	53	−42.4	−26.9	46.7	2	9	137
h46A10	46	500	0	25	40	−35.3	−1.7	23.3	1	63	60
h46A30/6	53	500	0	30	40	−42.1	−10.1	56.7	0	5	113
h53D2	46	500	0.1	25	48	−36.5	−14.5	40	2	30	157
h46A5	53	500	0.2	25	36	−33.9	−7.9	53.3	0	10	113
h53A6	53	500	0.3	25	48	−35.3	−8.5	43.3	2	138	49
h53B2	53	500	0.6	25	48	−30.1	−11.3	23.3	1	108	79
h46A11	46	500	0.6	25	20	−24.5	−1.5	43.3	0	0	143
h46A30/8	46	500	1.5	30	30	−34.2	−1.8	46.7	0	0	136
h45A30/7	45	500	1.6	30	50	−46.1	−4.7	73.3	0	0	158
h45A30/8	45	500	1.6	30	40	−39.3	−13.7	53.3	1	76	70
h53A3	53	500	2	25	56	−36.7	−13.7	36.7	0	147	40
h46A9	46	500	2.1	25	28	−31.5	−7.6	36.7	1	109	14
h53B3	53	500	3	25	48	−34.5	−5.5	48	2	98	89
h53D3	53	500	3.7	25	36	−34.3	−11.2	40	1	18	169
h44B30/8	44	500	4.6	30	37	−28.3	−23.5	40	1	34	84
h44B30/4	44	50	5	30	43	−38.2	−14.6	40	0	54	64
h46A6	46	100	5.4	25	36	−31.5	−8	46.7	1	0	123
h46A8	46	500	5.4	25	32	−28.6	0	20	1	76	47
h45A30/3	45	500	6.3	30	40	−35.5	−11.8	60	1	108	38
h53D5	53	500	7.9	25	36	−31.5	−3.3	66.7	1	0	187
h46A1	46	100	8.3	25	48	−35.7	−11.9	53.3	1	38	85
h53A5	53	250	9	25	48	−35.5	−8.5	43.3	2	141	46
h46A7	46	500	9.1	25	32	−34.8	−5.6	36.7	1	123	0
h53A30/5	53	100	9.4	30	47	−42.4	−11.3	46.7	1	141	41
h53A2	53	100	9.7	25	56	−36.1	−17.4	46.7	1	150	37
h53A4	53	500	10.5	25	48	−34.3	−8.5	20	0	144	43
h45A30/5	45	500	11.2	30	63	−44	−21.1	26.7	0	17	129
h53D4	53	500	12.3	25	32	−30.9	−9.2	63.3	1	6	181
h53A1	53	100	12.7	25	52	−38.6	−17.4	50	2	153	34
A25	51	250	14.9	25	36	−29.3	−11.6	66.7	2	146	62
h46A2	46	500	15.6	25	44	−31.2	−10.6	56.7	1	33	90
h46A30/7	46	500	18.5	30	30	−34.2	−6.2	53.3	1	0	141
h46A4	46	100	21.2	25	44	−39.9	−6.3	56.7	2	20	103
h44C30/2	44	50	22	30	33	−38	−7.4	36.7	1	7	111
h44B30/7	44	100	26	30	37	−33.9	−10.9	26.7	1	39	79
h51A	51	500	26.3	30	43	−40.3	−15	70	1	137	65
h44B30/6	44	500	32.5	30	37	−34.6	−9.6	30	2	44	74
h44C30/3	44	500	35	30	33	−38.9	−13.8	30	1	2	116
h44B30/1	44	100	35	30	33	−35.2	−7.1	66.7	1	69	49

TABLE 1-continued

Table 1: Table summarizing the characteristics of PMOs used

h53A30/6	53	500	35.9	30	47	−42.3	−8.5	56.7	1	338	44
h53A30/4	53	100	38.6	30	50	−43.4	−17.4	43.3	1	144	38
h44C30/1	44	100	42	30	37	−41.1	−10.4	50	1	12	106
h46A3	46	100	49.7	25	48	−43.1	−5.2	56.7	2	28	95
h44A30/3	44	250	52.1	30	37	−42.5	−8.6	56.7	1	99	19
h53A30/1	53	100	52.4	30	50	−48.1	−17.4	56.7	1	153	29
h44B30/3	44	500	61	30	43	−35.4	−11.4	30	0	59	59
h44B30/5	44	500	63.3	30	40	−35.9	−14.6	30	1	49	69
h45A30/1	45	500	64.5	30	60	−49.7	−11	36.7	1	146	0
h46A30/3	46	500	74.6	30	43	−49.8	−6.1	73.3	2	23	95
h46A30/1	46	500	75.6	30	47	−43.5	−12.3	63.3	0	33	85
h46A30/5	46	500	76.7	30	40	−49.2	−6.3	70	1	15	103
h53A30/3	53	100	80.1	30	53	−44.6	−17.4	53.3	1	147	35
h44B30/2	44	500	80.5	30	37	−36.9	−10.7	50	1	64	54
h53A30/2	53	100	87.2	30	53	−45.1	−17.4	63.3	1	150	32
h46A30/4	46	500	87.3	30	40	−47.5	−6.3	73.3	2	20	98
h46A30/2	46	500	87.9	30	47	−49.1	−13.4	63.3	2	28	90
h45A30/2	45	500	91.4	30	60	−46.6	−13	20	1	142	4
h44A30/2	44	500	95	30	43	−44	−8.6	40	0	104	14
h44A30/1	44	250	97	30	47	−47.5	−11.2	46.7	1	109	9

PMO	% overlap with hybrid. peak	% Rescue ESE sites	% overlap with Rescue ESE	% overlap with PESE	% overlap with PESS	ESE finder values over threshold[c] SF2/ASF	BRCA1	SC35	SRp40	SRp55	Tra2B	9G8
h53B1	0	5	56	40	40	0	9.26	3.62	10.66	0	5.06	1.1
h53C1	0	6	52	72	0	4.19	6.72	0	2.04	0	24.04	28.68
h53C2	0	1	24	60	0	4.19	6.72	10.2	4.38	0	0	8.28
h53C3	0	1	24	32	0	3.49	6.41	10.2	4.38	6.86	0	14.18
h53D1	0	4	40	32	0	0.52	0	18.68	0	6.86	0	12.71
h45A30/4	100	4	40	0	0	6.29	4.8	5.9	17.91	0	18.18	8.14
h45A30/6	100	4	40	0	0	11.64	7.34	5.04	1.38	0	7.25	16.53
h46A10	0	7	60	48	8	2.21	0	2.7	2.88	0	5.11	23.85
h46A30/6	0	7	40	50	0	0	0	0	5.09	0	24.04	6.94
h53D2	0	6	44	32	0	0.52	1.8	18.68	0.42	0	0	12.71
h46A5	0	7	48	44	0	0	0	0	5.09	0	24.04	6.94
h53A6	92	2	36	28	32	6.58	7.26	0	0	0	7.25	11.9
h53B2	0	5	60	60	0	0	9.26	3.62	4.73	0	5.06	8.28
h46A11	0	2	36	12	52	0	0	0	1.02	0	0	2.04
h46A30/8	0	1	27	27	43	0	0	0	1.02	0	0	2.04
h45A30/7	100	9	47	0	0	6.34	7.34	0	0.6	0	18.18	8.14
h45A30/8	100	4	47	0	0	0	0	5.9	2.4	0	18.18	17.14
h53A3	0	3	32	60	0	6.58	7.26	0	3.12	0	7.25	11.9
h46A9	0	8	48	25	0	0	7.87	0	0	0	24.04	7.14
h53B3	0	8	72	64	0	3.49	9.26	3.44	4.73	0	24.04	28.68
h53D3	0	9	64	0	0	0	1.8	0	6.95	0	24.04	10.49
h44B30/8	0	7	57	27	13	2.85	8.64	7.06	1.38	0	10.92	19.02
h44B30/4	0	8	47	37	27	1.98	8.64	6.14	10.12	0	7.25	8.28
h46A6	0	7	72	64	0	0	0	0	5.09	0	24.04	6.94
h46A8	0	5	56	24	60	2.21	0	3.56	2.88	0	0	23.68
h45A30/3	100	9	87	30	0	0	6.18	3.07	4.73	0.45	24.04	28.68
h53D5	0	14	92	44	0	8.5	11.95	0	7.67	0.33	24.04	7.14
h46A1	100	3	20	40	0	2.62	20.26	6.63	6.17	0	0	5.12
h53A5	100	3	36	36	20	6.58	7.26	0	3.12	0	7.25	11.9
h46A7	0	9	64	44	0	0	0	6.02	4.2	0	24.04	28.68
h53A30/5	100	5	47	47	17	6.58	7.26	0	3.12	0	7.25	11.9
h53A2	100	4	32	72	0	6.58	7.26	0	3.12	0	7.25	19.02
h53A4	100	4	28	48	8	6.58	7.26	0	3.12	0	7.25	11.9
h45A30/5	100	2	23	0	0	11.64	13.49	5.04	1.38	0	7.25	16.53
h53D4	0	16	96	24	0	8.5	11.95	0	7.67	0.33	24.04	7.14
h53A1	92	7	56	84	0	6.58	7.26	0	3.12	0	24.04	19.02
A25	0	1	24	12	32	1.22	13.72	0	0	0	0	0
h46A2	100	5	40	40	0	2.62	20.26	6.63	6.17	00	13.11	5.12
h46A30/7	0	2	20	10	43	0	0	0	1.02	0	0	2.1
h46A4	46	8	60	40	0	0	0	0	5.09	0	24.04	6.94
h44C30/2	0	3	33	10	63	0.52	5.72	0	0	0	9.46	5.6
h44B30/7	0	6	40	30	27	2.85	8.64	7.06	1.38	0	10.92	19.02
h51A	0	2	40	3	27	1.22	13.72	0	0	0	0	4.45
h44B30/6	0	8	37	20	27	2.85	8.64	0	1.92	0	10.92	19.02
h44C30/3	0	2	33	0	63	0	0	0	6.44	0	9.46	5.6
h44B30/1	0	6	67	33	30	0	0	6.14	10.12	0	10.92	8.28
h53A30/6	100	5	48	37	27	6.58	7.26	0	3.12	0	7.25	11.9
h53A30/4	100	4	43	57	7	6.58	7.26	0	3.12	0	7.25	11.9

TABLE 1-continued

Table 1: Table summarizing the characteristics of PMOs used

h44C30/1	0	3	43	27	63	0.52	5.72	7.06	0	0	9.46	5.6
h46A3	100	5	40	40	0	2.62	20.26	6.03	6.17	0	13.11	5.12
h44A30/3	0	3	23	0	77	0	13.26	0	0	0	0	11.3
h53A30/1	92	9	60	86	0	6.58	7.26	0	3.12	0	24.04	19.02
h44B30/3	0	5	47	37	33	0	0	6.14	10.12	0	7.25	8.28
h44B30/5	0	10	63	37	27	1.98	8.64	6.14	1.92	0	10.92	19.02
h45A30/1	100	2	0	0	6.7	3.43	8.64	5.16	3.54	3.57	0	20.56
h46A30/3	100	5	40	13	0	0	0.57	0	6.17	0	13.11	5.12
h46A30/1	100	5	33	33	0	2.62	20.26	6.63	6.17	0	13.11	5.12
h46A30/5	46	12	67	50	0	0	0	0	5.09	0	24.04	6.94
h53A30/3	100	6	43	67	0	6.58	7.26	0	3.12	0	24.04	19.02
h44B30/2	0	5	50	37	37	0	0	6.14	10.12	0	7.25	8.28
h53A30/2	100	8	53	77	0	6.58	7.26	0	3.12	0	24.04	19.02
h46A30/4	85	8	50	43	0	0	0.57	0	5.09	0	24.04	5.12
h46A30/2	100	5	33	33	0	2.62	20.26	6.63	6.17	0	13.11	5.12
h45A30/2	100	0	0	0	20	3.43	10.41	5.16	3.54	3.57	0	20.56
h44A30/2	0	3	27	0	63	0	13.26	0	0	0	0	11.3
h44A30/1	0	4	43	0	47	0	13.26	0	2.76	0	0	11.3

PMOs are ranked in order of efficacy and characteristics of the PMOs and their target sites listed.

[a]calculated as % skipped amplicon relative to total amplicon (i.e. skipped plus full length) as assessed by densitometric analysis of RT-PCR gels.

[b]calculated as % on PMO target site in open structures on predicted RNA secondary structure obtained using MFOLD analysis. The position of the PMO target sites relative to open loops in the RNA secondary structure is listed (0 = no ends in open loops, 1 = one end in an open loop, 2 = both ends in open loops).

[c]In analyses, SR binding sites were predicted using splice sequence finder (http://www.umd.be/SSF/) software. Values above threshold are given for PMOs whose target sites cover 50% or more of potential SR binding sites for SF2/ASF, BRCA1, SC35, SRp40, SRp55, Tra2β and 9G8.

TABLE 2

Table 2: The correlation of significant design parameters and PMO target site properties to skipping efficacy

Comparison	PMO-target binding energy	% open conformation	Length	Distance from acceptor site	% overlap with hybridisation peak	% overlap with strongest hybrid. peak	% overlap with BRCA1 motif
Ineffective vs Effective	0.001	0.094	0.017	0.004	0.056	0.003	0.026
Ineffective vs 5-25% skip	0.534	0.288	1	0.163	0.107	0.034	0.205
Ineffective vs 25-50% skip	0.02	0.316	0.014	0.067	0.614	0.195	0.079
Ineffective vs 50-75% skip	0.002	0.438	0.012	0.005	0.352	0.084	0.341
Ineffective vs 75-100% skip	<0.001	0.025	0.002	0.003	0.045	0.002	0.091
Ineffective vs >50% skip	<0.001	0.052	<0.001	<0.001	0.05	0.005	0.046
Spearmans correlation coefficient	−0.618	0.275	0.545	−0.421	0.258	0.46	0.261
Spearmans p value	0	0.0259	0	0	0.0363	0	0.0341

To establish the significance of design parameters and PMO target site properties to bioactivity, Mann-Whitney rank sum test analysis was performed for each, comparing ineffective (inactive) PMOs to the different groups of PMOs, subdivided (in the column headed "Comparison") according to bioactivity (efficacy). Criteria with p-values less than 0.05 in one or more comparisons are shown. The correlation of these variables to bioactivity is confirmed by Spearman rank order test analysis, for which Spearman correlation coefficients and p-values are given.

TABLE 3

Table 3: Linear discriminant analysis of effective and ineffective PMOs

Group	Classification: Effective	Ineffective	Total	Error rate	Average score
Effective	40	4	44	0.09	0.741
Ineffective	9	13	22	0.41	0.512
0-5% skip	9	13	22	0.41	0.512
5-25% skip	16	4	20	0.2	0.621
25-50% skip	9	0	9	0	0.806
50-75% skip	6	0	6	0	0.827
75-100% skip	10	0	10	0	0.857

Linear discriminant analysis [34] was used to predict the classification of PMOs on the basis of their PMO-target binding energy, overlap of PMO target site with a hybridization peak, and overlap of PMO target site with an ASF/SF2 (BRCA1) motif. PMOs have been grouped on the basis of their experimental bioactivity ("Group" column), and PMOs within each group predicted as "Effective" (bioactive) or "Ineffective" (inactive), as indicated by the column headings, according to the parameters used in the statistical analysis. The error rate for wrongly classifying a PMO, and the average score are given for each subgroup of PMO.

Example 2

[0074] Here, the inventors show the comparative analysis of a series of PMOs targeted to exon 53, skipping of which would have the potential to treat a further 8% of DMD patients with genomic deletions on the Leiden database compared to skipping of exon 51 which has the potential to treat 13% of DMD patients [37]. An array of overlapping PMOs were designed for the targeting of exon 53 as described previously [38]. These were all tested initially in normal human skeletal muscle cells (hSkMCs), since these are more readily available than patient cells. PMOs that showed greatest skipping efficacy were further tested in cells from a DMD patient with a relevant deletion (del 45-52). The PMOs with greatest efficacy, in terms of concentration and stability, were evaluated by performing dose-response and time-course studies. Findings from these experiments were supported by in vivo studies in a mouse model transgenic for the entire human dystrophin locus [8]. Collectively, this work suggests that one particular PMO (A, h53A30/1, +30+59) produced the most robust skipping of exon 53, and should be considered the sequence of choice for any upcoming PMO clinical trial.

Materials and Methods

AO Design

[0075] Twenty-three overlapping AOs to exon 53 were designed as described above in Example 1.

Cell Culture and AO Transfection

[0076] Transfections were performed in two centres (Royal Holloway, London UK (RHUL) and UCL Institute of Child Health, London UK (UCL)) and by two different methods (liposome-carrier of leashed PMOs in normal cells (RHUL), and by nucleofection of naked PMOs in patient cells (UCL)). AOs were transfected into normal human primary muscle cells (TCS Cellworks, Buckingham, UK) and into patient primary skeletal muscle cultures obtained from muscle biopsies taken at the Dubowitz Neuromuscular Unit, UCL Institute of Child Health (London, UK), with the approval of the institutional ethics committee. Normal hSkMCs were cultured and transfected with leashed PMOs, using 1:4 lipofectin, as described previously [4]. To minimize any influence of leash design on PMO uptake and subsequent bioactivity, the DNA sequences in the leashes were of the same length (17mers for the 25mer PMOs or 20mers for the 30mer PMOs) and were completely complementary to the 3'-most 17 or 25 nt of each PMO. The phosphorothioate caps of 5 nt at each end were not complementary to the PMOs, and had the same sequences for every leash.

DMD Patient Primary Myoblast Culture

[0077] Skeletal muscle biopsy samples were taken from a diagnostic biopsy of the quadriceps from a DMD patient with a deletion of exons 45-52. Informed consent was obtained before any processing of samples. Muscle precursor cells were prepared from the biopsy sample by sharp dissection into 1 mm^3 pieces and disaggregated in solution containing HEPES (7.2 mg/ml), NaCl (7.6 mg/ml), KCl (0.224 mg/ml) Glucose (2 mg/ml) Phenol Red (1.1 μg/ml) 0.05% Trypsin-0.02% EDTA (Invitrogen, Paisley, UK) in distilled water, three times at 37° C. for 15 minutes in Wheaton flasks with vigorous stirring. Isolated cells were plated in non-coated plastic flasks and cultured in Skeletal Muscle Growth Media (Promocell, Heidelberg, Germany) supplemented with 10% Foetal Bovine Serum (PAA Laboratories, Yeovil, UK), 4 mM L-glutamine and 5 μg/ml gentamycin (Sigma-Aldrich, Poole, UK) at 37° C. in 5% CO_2.

Nucleofection of DMD Primary Myoblasts

[0078] Between 2×10^5 and 1×10^6 cells/ml were pelleted and resuspended in 100 μl of solution V (Amaxa Biosystems, Cologne, Germany). The appropriate PMO to skip exon 53 was added to the cuvette provided, sufficient to give the concentrations described, followed by the cell suspension, and nucleofected using the Amaxa nucleofector 2, program B32. 500 μl of media was added to the cuvette immediately following nucleofection. This suspension was transferred to a 6 well plate in differentiation medium. Nucleofected cells were maintained in differentiation media for 3-21 days post treatment before extraction of RNA or protein.

Lactate Dehydrogenase Cytotoxicity Assay

[0079] A sample of medium was taken 24 hours post-transfection to assess cytotoxicity by release of lactate dehydrogenase (LDH) into the medium, using the LDH Cytotoxicity Detection Kit (Roche, Burgess Hill, UK), following the manufacturer's instructions. The mean of three readings for each sample was recorded, with medium only, untreated and dead controls. The readings were normalised for background (minus medium only) and percentage toxicity expressed as [(sample−untreated)/(dead−untreated)×100].

RNA Isolation and Reverse Transcription Polymerase Chain Reaction Analysis

[0080] As with cell culture, two different techniques were used in the two centres involved in this study for isolating RNA and its analysis by RT-PCR, as described previously [4]. PCR products were analysed on 1.5% (w/v) agarose gels in Tris-borate/EDTA buffer. Skipping efficiencies were determined by quantification of the full length and skipped PCR products by densitometry using GeneTools software (Syngene, Cambridge, UK).

Sequence Analysis

[0081] RT-PCR products were excised from agarose gels and extracted with a QIAquick gel extraction kit (Qiagen, Crawley, UK). Direct DNA sequencing was carried out by the MRC Genomics Core Facility.

Western Blot Analysis of Dystrophin Protein

[0082] DMD patient cells, transfected as described and cultured in differentiation medium, were harvested 7, 14 or 21 days post-transfection. 4×10^5 cells were pelleted and resuspended in 50 μl of loading buffer (75 mM Tris-HCl pH 6.8, 15% sodium dodecyl sulphate, 5% β-mercaptoethanol, 2% glycerol, 0.5% bromophenol blue and complete mini protease inhibitor tablet). Samples were incubated at 95° C. for 5 minutes and centrifuged at 18,000×g for 5 minutes. 20 μl of sample was loaded per well in a 6% polyacrylamide gel with 4% stacking gel. Protein from CHQ5B cells differentiated for 7 days was used as a positive control for dystrophin. Gels were electrophoresed for 5 hours at 100V before blotting on nitrocellulose membrane at 200 mA overnight on ice. Blots were stained with Protogold to assess protein loading, then blocked in 10% non-fat milk in PBS with 2% tween (PBST)

for 3 hours. Blots were probed with antibodies to dystrophin, NCL-DYS1 (Vector Labs, Peterborough, UK) diluted 1:40 and to dysferlin, Hamlet1 (Vector Labs) diluted 1:300 in 3% non-fat milk/PBST. An anti-mouse, biotinylated secondary antibody (diluted 1:2000; GE Healthcare, Amersham, UK) and streptavidin/horse radish peroxidise conjugated antibody (1:10,000; Dako, Ely, UK) allowed visualisation in a luminol-HRP chemiluminescence reaction (ECL-Plus; GE Healthcare) on Hyperfilm (GE Healthcare), exposed at intervals from 10 seconds to 4 minutes.

Transgenic Human DMD Mice

[0083] A transgenic mouse expressing a complete copy of the human DMD gene has been generated [8, 39]. Experiments were performed at the Leiden University Medical Center, with the authorization of the Animal Experimental Commission (UDEC) of the Medical Faculty of Leiden University as described previously [4].

Results

[0084] Twenty-three PMOs were designed to target exon 53, as described previously [38]. Briefly, SR protein binding motifs, RNA secondary structure and accessibility to binding as determined by hexamer hybridization array analysis, were used as aids to design (FIG. **1**). Table 4 summarises the names and target sequence characteristics of these PMOs. These PMOs were initially characterized in normal human skeletal muscle cells (at RHUL). The most active were then directly compared to the PMO targeting the sequence previously identified as most bioactive by Wilton et al. [19] in exon 53-skippable patient cells (at UCL), and in the humanised DMD mouse (at LUMC).

Comparison of PMOs to Exon 53 in Normal Human Skeletal Muscle Cells

[0085] An array of seventeen 25mer leashed PMOs were transfected, at a concentration of 500 nM, into normal human skeletal muscle myoblast cultures using lipofectin. Of these seventeen, only four produced consistent levels of exon skipping considered to be above background i.e. over 5% skipping [38], as assessed by densitometric analysis (FIG. **6***a*). These were PMO-A, -B, -C and -D, which targeted exon 53 at positions +35+59, +38+62, +41+65 and +44+68 respectively. The levels of exon skipping produced were as follows: PMO-A, 12.7%; PMO-B, 9.7%; PMO-C, 10.5%; and PMO-D, 9.0%. When nucleofection was used as a means of introducing naked PMOs into the cells, higher levels of exon skipping were observed for PMO-A and PMO-B only, with 300 nM doses producing 41.2% and 34.3% exon skipping, respectively. The superiority of nucleofection over lipofection has been observed by others (Wells et al., in preparation). However no exon skipping was evident following nucleofection with any of the other naked 25mer PMOs tested (data not shown).

[0086] A 3 nt-stepped array of 30mer PMOs was then designed to target the region of exon 53 (position +30 to +74) associated with exon skipping activity by the 25mer PMOs. Following lipofection into normal human skeletal muscle myoblast cultures at a concentration of 500 nM, PMO-G (+30+59), PMO-H (+33+62), PMO-I (+36+65), PMO-J (+39+68) and PMO-K (+42+71) gave reproducible exon skipping above background (FIG. **6***b*), while PMO-L (+45+74) was inactive. The levels of exon skipping produced were as follows: PMO-G, 37.1%; PMO-H, 44.5%; PMO-I, 27.4%; PMO-J, 33.0%; and PMO-K, 13.0%. The concentration dependence of exon skipping by the more active 30mer PMOs was examined further (FIG. **7***a*). PMO-H and PMO-I were able to produce convincing skipping at concentrations as low as 25 nM, while PMO-G was active at 50 nM and PMO-J at 75 nM. The exon skipping produced by these 30mer PMOs was shown to be persistent, surviving the lifetime of the cultures (14 days) (FIG. **7***b* and data not shown). When unleashed 30mer PMOs were introduced into normal muscle cultures by nucleofection, high levels of exon skipping were also observed. For example, at 300 nM, PMO-G and PMO-H gave over 80% skipping of exon 53 (data not shown).

Comparison of PMOs to Exon 53 in DMD Patient Cells

[0087] The PMOs, both 25mer and 30mer, that produced the highest levels of DMD exon 53 skipping in normal skeletal muscle cultures, were then compared to each other for bioactivity in DMD patient (del 45-52) cells, and were also compared to an additional reagent, PMO-M (+39+69), described previously [19]. This comparative evaluation was performed in a blinded fashion. When tested and compared directly at 300 nM doses by nucleofection, PMO-G, PMO-H and PMO-A were most active producing in the order of 60% exon skipping (FIG. **8**). The other PMOs tested produced the following exon skipping levels: PMO-I, 45%; PMO-B, 41%; PMO-J, 27%; PMO-M, 26%. All the other PMOs tested gave exon skipping at lower levels of between 10 and 20%.

[0088] When the concentration dependence of exon skipping was examined for the most bioactive PMOs, levels approaching 30% were evident for PMO-G and PMO-H at concentrations as low as 25 nM (FIG. **9***a, b*). Similar levels of skipping were only achieved by PMO-A, PMO-B and PMO-M at 100 nM, while PMO-I needed to be present at 200 nM to produce over 30% exon skipping (FIG. **9***a, b*). There was no evidence that any of the PMOs tested caused cellular cytotoxicity relative to mock-transfected controls, as assessed by lactate dehydrogenase release into culture medium (results not shown). The exon skipping produced by the six most bioactive PMOs was shown to be persistent, lasting for up to 10 days after transfection, with over 60% exon skipping observed for the lifetime of the cultures for PMO-A, PMO-G and PMO-H (FIG. **10***a, b*). Exon skipping was shown to persist for 21 days for PMO-A and PMO-G (FIG. **10***c*).

[0089] Western blot analysis of DMD patient (del 45-52) cell lysates, treated in culture with the most bioactive 25mers (PMO-A and PMO-B) and longer PMOs (PMO-G, PMO-H, PMO-I and PMO-M) is shown in FIG. **10***e*. De novo expression of dystrophin protein was evident with all six PMOs, but was most pronounced with PMO-H, PMO-I, PMO-G and PMO-A, producing 50%, 45%, 33% and 26% dystrophin expression, respectively, relative to the positive control, and seemingly weakest with PMO-B and PMO-M (11% and 17% dystrophin expression respectively, relative to the positive control). However, the limitations of quantifying Western blots of this nature should be taken into account when interpreting the data.

Comparison of PMOs to Exon 53 in Humanised DMD Mouse

[0090] The hDMD mouse is a valuable tool for studying the processing of the human DMD gene in vivo, and as such provides a model for studying the in vivo action of PMOs,

prior to clinical testing in patients. PMO-A, PMO-G, PMO-H, PMO-I and PMO-M were injected into the gastrocnemius muscle of hDMD mice, and RNA extracted from the muscles was analysed for exon 53 skipping (FIG. **11**). Skipping of exon 53 is evident for each of the PMOs tested; 8% for PMO-A, 7.6% for PMO-I, 7.2% for PMO-G, but to a slightly lower level of 4.8% for PMO-H. PMO-M produced exon skipping levels of less than 1%, which is the detection threshold for the system used.

[0091] It should be noted that the levels of exon skipping by each particular PMO was variable. This has been reported previously [8], and is likely to be due to the poor uptake into the non-dystrophic muscle of the hDMD mouse. However this does not compromise the importance of the finding that the PMOs tested here are able to elicit the targeted skipping of exon 53 in vivo.

[0092] Of the 24 PMOs tested, six (PMO-A, PMO-B, PMO-G, PMO-H, PMO-I and PMO-M) produced over 50% targeted skipping of exon 53 either in normal myotubes or in patient myotubes or both. The characteristics of these active PMOs and their target sites are summarised in Table 4. They all showed strong overlap (92%-100%) with the sequence shown to be accessible to binding on the hybridization array analysis, had similar GC content (50%-56%), but varying degrees of overlap (32%-60%) with ESE sites as predicted by Rescue ESE analysis, varying degrees of overlap with ESE sites and ESS sites (60%-86% and 0%-10%, respectively) as predicted by PESX analysis, and all showed overlap with two SR binding motifs (SF2/ASF, as defined by the BRCA1 algorithm, and SRp40). It should be noted that PMO-J, -K, -L and M had a common SNP of exon 53 (c7728C>T) in the last, fourth to last, seventh to last and second to last base, respectively of their target sites. There is the potential that this allelic mismatch could influence the binding and bioactivity of these PMOs. However, the more active PMOs (-A, -B, -G, -H and -I) all had their target sites away from the SNP, and the possible effect of a mismatch weakening binding and bioactivity is removed, and allows definitive comparisons between these PMOs to be made.

Discussion

[0093] The putative use of AOs to skip the exons which flank out-of-frame deletions is fast becoming a reality in the experimental intervention of DMD boys. Indeed the restoration of dystrophin expression in the TA muscle of four patients, injected with a 2'OMePS AO optimised to target exon 51 of the DMD gene, has been reported recently [11]. Moreover a clinical trial using a PMO targeting exon 51 has recently been completed in seven DMD boys in the UK (Muntoni et al, in preparation). However, the targeted skipping of exon 51 would have the potential to treat only 13% of DMD patients with genomic deletions on the Leiden database [37]. There is therefore a definite requirement for the optimisation of AOs to target other exons commonly mutated in DMD.

[0094] Although there have been many large screens of AO bioactivity in vitro [18, 19, 38, 40], no definite rules to guide AO design have become apparent. Previous studies in the mdx mouse model of DMD showed that AOs that targeted the donor splice site of exon 23 of the mouse DMD gene restored dystrophin expression [7]. However the targeting of AOs to the donor splice sites of exon 51 of the human DMD gene was ineffective at producing skipping [4], and it has been suggested that the ‘skippability’ of human DMD exons has no correlation with the predicted strength of the donor splice site [41]. It has been reported that exon skipping could be induced by the targeting of AOs to exonic splicing enhancer (ESE) motifs [18, 40]. These motifs are recognised by SR proteins, which facilitate exon splicing by recruiting splicing effectors (U1 and U2AF) to the donor splice site (reviewed by Cartegni et al.) [42]. However these motifs are divergent, poorly defined, their identification complex, and their strength as AO design tools dubious [38].

[0095] A comparative study of 66 PMOs designed to five different DMD exons demonstrated the significance of RNA secondary structure in relation to accessibility of the PMO target site and subsequent PMO bioactivity [38], as assessed by mfold software prediction of secondary structure [25], and a hybridization screen against a hexamer array [38]. PMOs that bound to their target more strongly, either as a result of being longer or in being able to access their target site more directly, were significantly more bioactive. The influence of AO length on bioactivity has been reported elsewhere [4, 30], and is further confirmed in the present study; all 30mers tested were more bioactive relative to their 25mer counterpart. The fact that 30mer PMOs were more bioactive than 25mer PMOs targeted to the same open/accessible sites on the exon, would suggest that strength of binding of PMO to the target site may be the most important factor in determining PMO bioactivity. These thermodynamic considerations have also been reported in a complementary study of 2'OMePS AOs [40]. However, it has also been reported that two overlapping 30mers were not as efficient as a 25mer at skipping mouse exon 23, indicating that oligomer length may only be important in some cases [4].

[0096] To ensure that the analysis of PMOs for the targeted skipping of exon 53 was not biased by any particular design strategy, seventeen 25mer PMOs were designed to cover the whole of exon 53, with stepwise arrays over suggested bioactive target sites, and then subsequently six 30mer PMOs were designed to target the sequence of exon 53 that showed an association with exon skipping for the 25mers tested. PMOs were designed and tested independently by two different groups (at RHUL and UWA), and then efficacy of the best thirteen sequences confirmed by two other independent groups (at UCL and LUMC). Such a collaborative approach has been used previously as a way of validating target sequences in DMD [4]. Human myoblasts allowed the controlled in vitro comparison of PMO sequences, and confirmation of skipping of exon 53 at the RNA level by certain PMOs in both normal cells and, perhaps more importantly, in DMD patient cells with a relevant mutation. These results were further borne out by the expression of dystrophin protein in the DMD cells treated with specific PMOs. Use of the humanised DMD mouse provided an in vivo setting to confirm correct exon exclusion prior to any planned clinical trial. The combined use of these three different systems (normal cells, patient cells and hDMD mouse) as tests of PMO bioactivity provided a reliable and coherent determination of optimal sequence(s) for the targeted skipping of exon 53.

[0097] When considering the data presented here as a whole, the superiority of the PMO targeting the sequence +30+59 (PMO-G, or h53A30/1), is strongly indicated. In normal myoblasts, nucleofection of PMO-G (300 nM) and liposomal-carrier mediated transfection of leashed PMO-G (500 nM) produced over 80% and over 50% skipping of exon 53, respectively, implying that it acts extremely efficiently within the cell. This was confirmed in patient cells. Indeed, this PMO generates the highest levels of exon skipping in

patient cells over a range of concentrations (up to 200 nM) and, most important therapeutically, exerts its activity at concentrations as low as 25 nM. The exon skipping activity of this PMO is also persistent, with over 70% exon skipping for 7 days in culture, and over 60% exon skipping for up to three weeks. This would have important safety and cost implications as a genetic therapy for DMD patients with the appropriate deletions. PMO-G was also shown to skip exon 53 correctly in vivo. These RNA results were further confirmed by the detection of dystrophin protein at a high level in protein extracts from patient cells treated with PMO-G. Previous studies by the Leiden group [18] suggest that the optimal 2'OMePS AO is targeted to the sequence +46+63 of exon 53, producing exon skipping in up to 25% of transcripts in cultured cells and 7% in the hDMD mouse. This 2'OMePS AO shows some degree of overlap with the optimal PMOs reported here which strengthens our findings. The reason that our optimal PMO is more specific could be a (combined) consequence of the different AO chemistries, length of AO used, and the absolute target site of AO.

[0098] The sequence h53A30/1 we have identified appears to be more efficient than any of the previously reported AOs designed to skip exon 53 of the DMD gene, and this PMO therefore represents, at the present time, the optimal sequence for clinical trials in DMD boys.

TABLE 4

Table 4: Table summarizing the characteristics of PMOs used

	PMO	Position Start	Position End	% GC	Exon-PMO binding energy	PMO-PMO binding energy	% open[b]	Ends in open loops[b]	% overlap with hybrid. peak	# Rescue ESE sites	% overlap with Rescue ESE
A	h53A1	+35	+59	52	−38.6	−17.4	50	2	92	7	56
B	h53A2	+38	+62	56	−36.1	−17.4	46.7	1	100	4	32
C	h53A3	+41	+65	56	−36.7	−13.7	36.7	0	0	3	32
D	h53A4	+44	+68	48	−34.3	−8.5	20	0	100	4	28
E	h53A5	+47	+71	48	−35.5	−8.5	43.3	2	100	3	36
F	h53A6	+50	+74	48	−35.3	−8.5	43.3	2	92	2	36
N	h53B1	+69	+93	28	−22.1	−12.1	53.3	1	0	5	56
O	h53B2	+80	+104	48	−30.1	−11.3	23.3	1	0	5	60
P	h53B3	+90	+114	48	−34.5	−5.5	48	2	0	8	72
Q	h53C1	+109	+133	48	−32.4	−9.8	46.7	2	0	6	52
R	h53C2	+116	+140	56	−31.3	−12.7	33.3	1	0	1	24
S	h53C3	+128	+152	60	−34.6	−13.7	26.7	1	0	1	24
T	h53D1	+149	+173	52	−34.1	−13.4	30	1	0	4	40
U	h53D2	+158	+182	48	−36.5	−14.5	40	2	0	6	44
V	h53D3	+170	+194	36	−34.3	−11.2	40	1	0	9	64
W	h53D4	+182	+206	32	−30.9	−9.2	63.3	1	0	16	96
X	h53D5	+188	+212	36	−31.5	−3.3	66.7	1	0	14	92
G	h53A30/1	+30	+59	50	−48.1	−17.4	56.7	1	92	9	60
H	h53A30/2	+33	+62	53	−45.1	−17.4	63.3	1	100	8	53
I	h53A30/3	+36	+65	53	−44.6	−17.4	53.3	1	100	6	43
J	h53A30/4	+39	+68	50	−43.4	−17.4	43.3	1	100	4	43
K	h53A30/5	+42	+71	47	−42.4	−11.3	46.7	1	100	5	47
L	h53A30/6	+45	+74	47	−42.3	−8.5	56.7	1	100	5	48
M	H53A	+39	+69	52	−48.5	−17.4	48.4	2	100	4	45

	PMO	% overlap with PESE	% overlap with PESS	ESE finder values over threshold[c] SF2/ASF	BRCA1	SC35	SRp40	SRp55	Tra2B	9G8
A	h53A1	84	0	6.58	7.26	0	3.12	0	24.04	19.02
B	h53A2	72	0	6.58	7.26	0	3.12	0	7.25	19.02
C	h53A3	60	0	6.58	7.26	0	3.12	0	7.25	11.9
D	h53A4	48	8	6.58	7.26	0	3.12	0	7.25	11.9
E	h53A5	36	20	6.58	7.26	0	3.12	0	7.25	11.9
F	h53A6	28	32	6.58	7.26	0	0	0	7.25	11.9
N	h53B1	40	40	0	9.26	3.62	10.66	0	5.06	1.1
O	h53B2	60	0	0	9.26	3.62	4.73	0	5.06	8.28
P	h53B3	64	0	3.49	9.26	3.44	4.73	0	24.04	28.68
Q	h53C1	72	0	4.19	6.72	0	2.04	0	24.04	28.68
R	h53C2	60	0	4.19	6.72	10.2	4.38	0	0	8.28
S	h53C3	32	0	3.49	6.41	10.2	4.38	6.86	0	14.18
T	h53D1	32	0	0.52	0	18.68	0	6.86	0	12.71
U	h53D2	32	0	0.52	1.8	18.68	0.42	0	0	12.71
V	h53D3	0	0	0	1.8	0	6.95	0	24.04	10.49
W	h53D4	24	0	8.5	11.95	0	7.67	0.33	24.04	7.14
X	h53D5	44	0	8.5	11.95	0	7.67	0.33	24.04	7.14
G	h53A30/1	86	0	6.58	7.26	0	3.12	0	24.04	19.02
H	h53A30/2	77	0	6.58	7.26	0	3.12	0	24.04	19.02
I	h53A30/3	67	0	6.58	7.26	0	3.12	0	24.04	19.02

TABLE 4-continued

Table 4: Table summarizing the characteristics of PMOs used

J	h53A30/4	57	7	6.58	7.26	0	3.12	0	7.25	11.9
K	h53A30/5	47	17	6.58	7.26	0	3.12	0	7.25	11.9
L	h53A30/6	37	27	6.58	7.26	0	3.12	0	7.25	11.9
M	H53A	58	10	6.58	7.26	0	3.12	0	7.25	11.9

Characteristics of the PMOs and their target sites listed.

[b]calculated as % of PMO target site in open structures on predicted RNA secondary structure obtained using MFOLD analysis. The position of the PMO target sites relative to open loops in the RNA secondary structure is listed (0 = no ends in open loops, 1 = one end in an open loop, 2 = both ends in open loops).

[c]In the analyses, SR binding sites were predicted using splice sequence finder (http://www.umd.be/SSF/) software. Values above threshold are given for PMOs whose target sites cover 50% or more of potential binding sites for SF2/ASF, BRCA1, SC35, SRp40, SRp55, Tra2β and 9G8

REFERENCES

[0099] 1. Hoffmann E P, Brown R H, Kunkel L M (1987) Dystrophin: The protein product of the Duchenne muscular dystrophy locus. *Cell;* 51: 919-928.

[0100] 2. Den Dunnen J T, Grootsscholten P M, Bakker E, Blonden L A, Ginjaar H B, Wapenaar M C, et al. (1989). Topography of the Duchenne muscular dystrophy (DMD) gene: FIGE and cDNA analysis of 194 cases reveals 115 deletions and 13 duplications. *Am J Hum Genet;* 45: 835-847.

[0101] 3. van Deutekom J C, Bremmer-Bout M, Janson A A, Ginjaar I B, Baas F, den Dunnen J T, et al. (2001). Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum Mol Genet;* 10: 1547-1554.

[0102] 4. Arechavala-Gomeza V, Graham I R, Popplewell L J, Adams A M, Aartsma-Rus A, Kinali M, et al. (2007). Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during pre-mRNA splicing in human muscle. *Hum Gene Ther;* 18: 798-810.

[0103] 5. Mann C J, Honeyman K, Cheng A J, Ly T, Lloyd F, Fletcher S, et al. (2001). Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc Natl Acad Sci USA;* 98: 42-47.

[0104] 6. Lu Q L, Mann C J, Lou F, Bou-Gharios G, Morris G E, Xue S A, et al. (2003). Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nat Med;* 9: 1009-1014.

[0105] 7. Graham I R, Hill V J, Manoharan M, Inamati G B, Dickson G (2004). Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligonucleotides (splicomers): target sequence optimisation using oligonucleotide arrays. *J Gene Med;* 6: 1149-1158.

[0106] 8. Bremmer-Bout M, Aartsma-Rus A, de Meijer E J, Kaman W E, Janson A A, Vossen R H, et al. (2004). Targeted exon skipping in transgenic hDMD mice: A model for direct preclinical screening of human-specific antisense oligonucleotides. *Mol Ther;* 10: 232-240

[0107] 9. Jearawiriyapaisarn N, Moulton H M, Buckley B, Roberts J, Sazani P, Fucharoen S, et al. (2008). Sustained dystrophin expression induced by peptide-conjugated morpholino oligomers in the muscles of mdx mice. *Mol. Ther*. June 10 (Epub).

[0108] 10. Bertoni C. (2008). Clinical approaches in the treatment of Duchenne muscular dystrophy (DMD) using oligonucleotides. *Front Biosci;* 13: 517-527.

[0109] 11. van Deutekom J C, Janson A A, Ginjaar I B, Franzhuzen W S, Aartsma-Rus A, Bremmer-Bout M, et al. (2007). Local antisense dystrophin restoration with antisense oligonucleotide PRO051. *N Eng J Med;* 357: 2677-2687.

[0110] 12. Gebski B L, Mann C J, Fletcher S, Wilton S D (2003). Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. *Hum Mol Genet;* 12: 1801-1811.

[0111] 13. Alter J, Lou F, Rabinowitz A, Yin H, Rosenfeld J, Wilton S D, et al. (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med;* 12: 175-177.

[0112] 14. Fletcher S, Honeyman K, Fall A M, Harding P L, Johnsen R D, Wilton S D (2006). Dystrophin expression in the mdx mouse after localized and systemic administration of a morpholino antisense oligonucleotide. *J Gene Med;* 8: 207-216.

[0113] 15. McClorey G, Fall A M, Moulton H M, Iversen P L, Rasko J E, Ryan M, et al. (2006). Induced dystrophin exon skipping in human muscle explants. *Neuromus Disorders;* 16: 583-590.

[0114] 16. McClorey G, Moulton H M, IversenPL, Fletcher S, Wilton S D (2006). Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. *Gene Ther;* 13:1373-1381.

[0115] 17. Arora V, Devi G R, Iversen P L (2004). Neutrally charged phosphorodiamidate morpholino antisense oligomers: uptake, efficacy and pharmacokinetics. *Curr Pharm Biotechnol;* 5: 431-439.

[0116] 18. Aartsma-Rus A, De Winter C L, Janson A A M, Kaman W E, van Ommen G-JB, Den Dunnen J T, et al. (2005). Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: Indication for steric hindrance of SR protein binding sites. *Oligonucleotides;* 15: 284-297.

[0117] 19. Wilton S D, Fall A M, Harding P L, McClorey G, Coleman C, Fletcher S (2007). Antisense oligonucleotide-induced exon skipping across the human dystrophin gene transcript. *Mol Ther;* 15: 1288-1296.

[0118] 20. Cartegni L, Wang J, Zhu Z, Zhang M Q, Krainer A R (2003). ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res;* 31: 3568-3571.

[0119] 21. Smith P J, Zhang C, Wang J, Chew S L, Zhang M O, Krainer A R (2006). An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers. *Human Mol Genet;* 15: 2490-2508.

[0120] 22. Zhang X H, Chasin L H (2004). Computational definition of sequence motifs governing constitutive exon splicing. *Genes Dev;* 18: 1241-1250.

[0121] 23. Zhang X H, Leslie C S, Chasin L A (2005). Computational searches for splicing signals. *Methods;* 37: 292-305.

[0122] 24. Fairbrother W G, Yeh R F, Sharp P A, Burge C B (2002). Predictive identification of exonic splicing enhancers in human genes. *Science;* 297: 1007-1013.

[0123] 25. Mathews D H, Sabina J, Zuker M, Turner D H (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J Mol Biol;* 288: 911-940.

[0124] 26. Aartsma-Rus A, Bremmer-Bout M, Janson A A M, den Dunnen J T, van Ommen G-JB, van Deutekom J C T (2002). Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. *Neuromus Disorders;* 12: 871-877.

[0125] 27. Aartsma-Rus A, Kaman W E, Weij R, den Dunnen J T, van Ommen G J, van Deutekom J C. (2006). Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons. *Mol Ther;* 14: 401-407.

[0126] 28. Adams A M, Harding P L, Iversen P L, Coleman C, Fletcher S, Wilton S D. (2007). Antisense oligonucleotide induced exon skipping and the dystrophin gene transcript: cocktails and chemistries. *BMC Mol Biol;* 8: 57.

[0127] 29. Vickers T A, Wyatt J R, Freier S M (2000). Effects of RNA secondary structure on cellular antisense activity. *Nucleic Acids Res;* 28: 1340-1347.

[0128] 30. Harding P L, Fall A M, Honeyman K, Fletcher S, Wilton S D (2007). The influence of antisense oligonucleotide length on dystrophin exon skipping. *Mol Ther;* 15: 157-166.

[0129] 31. Wee K B, Pramono Z A D, Wang J L, MacDorman K F, Lai P S, Yee WC (2008). Dynamics of co-translational pre-mRNA folding influences the induction of dystrophin exon skipping by antisense oligonucleotides. *Plos one;* 3: e1844.

[0130] 32. Fairbrother W G, Yeo G W, Yeh R, Goldstein P, Mawson M, Sharp P A, et al. (2004). RESCUE-ESE identifies candidate exonic splicing enhancers in vertebrate exons. *Nucleic Acids Res;* 32: W187-190.

[0131] 33. Patzel V, Steidl R, Kronenwell R, Haas R, Sczakiel G (1999). A theoretical approach to select effective antisense oligodeoxyribonucleotides at high statistical probability. *Nucleic Acids Res;* 27: 4328-4334.

[0132] 34. Ihaka R, Gentleman R C (1996). R: A Language for Data Analysis and Graphics. *Journal of Computational and Graphical Statistics;* 15: 999-1013.

[0133] 35. Moulton H M, Fletcher S, Neuman B W, McClorey G, Stein D A, Abes S, Wilton S D, Buchmeier M J, Lebleu B, Iversen P L (2007). Cell-penetrating peptide-morpholino conjugates alter pre-mRNA splicing of DMD (Duchenne muscular dystrophy) and inhibit murine coronavirus replication in vivo. *Biochem. Soc. Trans.* 35: 826-8.

[0134] 36. Jearawiriyapaisarn N, Moulton H M, Buckley B, Roberts J, Sazani P, Fucharoen S, Iversen P L, Kole R (2008). Sustained Dystrophin Expression Induced by Peptide-conjugated Morpholino Oligomers in the Muscles of mdx Mice. *Mol. Ther.* June 10. Epub ahead of print.

[0135] 37. Aartsma-Rus A, Fokkema I, Verschuuren J, Ginjaar I, van Deutekom J, van Ommen G J et al. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum Mutation* 2009; Jan. 20 (Epub).

[0136] 38. Popplewell L J, Trollet C, Dickson G, Graham I R. Design of phosphorodiamidate morpholino oligomers (PMOs) for the induction of exon skipping of the human DMD gene. *Mol Ther* 2009; Jan. 13 (Epub).

[0137] 39. 'tHoen PAC, de Meijer E J, Boer J M, Vossen R H, Turk R, Maatman R G et al. (2008) Generation and characterization of transgenic mice with the full-length human DMD gene. *J Biol Chem;* 283: 5899-5907.

[0138] 40. Aartsma-Rus A, van Vliet L, Hirschi M, Janson A A, Heemskerk H, de Winter C L, et al. Guidelines for antisense oligonucleotide design and insight into splice-modulating mechanisms. *Mol Ther* 2008; Sep. 23 (Epub).

[0139] 41. Aartsma-Rus A, van Ommen G J. Antisense-mediated exon skipping: A versatile tool with therapeutic and research applications. *RNA* 2007; 13: 1-16.

[0140] 42. Cartegni L, Chew S L, Krainer A R. Listening to silence and understanding nonsense: Exonic mutations that affect splicing. *Nat Rev Genet.* 2002; 3: 285-298.

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```
                                            (SEQ ID NO: 1)
a)   XGA AAA CGC CGC CAX XXC XCA ACA GAX CXG;

                                            (SEQ ID NO: 2)
b)   CAX AAX GAA AAC GCC GCC AXX XCX CAA CAG;

                                            (SEQ ID NO: 3)
c)   XGX XCA GCX XCX GXX AGC CAC XGA XXA AAX;

                                            (SEQ ID NO: 4)
d)   CAG XXX GCC GCX GCC CAA XGC CAX CCX GGA;

                                            (SEQ ID NO: 5)
e)   XXG CCG CXG CCC AAX GCC AXC CXG GAG XXC;

                                            (SEQ ID NO: 6)
f)   XGC XGC XCX XXX CCA GGX XCA AGX GGG AXA;
```

-continued

```
                                            (SEQ ID NO: 7)
g)   CXX XXA GXX GCX GCX CXX XXC CAG GXX CAA;

                                            (SEQ ID NO: 8)
h)   CXX XXC XXX XAG XXG CXG CXC XXX XCC AGG;

                                            (SEQ ID NO: 9)
i)   XXA GXX GCX GCX CXX XXC CAG GXX CAA GXG;

                                           (SEQ ID NO: 10)
j)   CXG XXG CCX CCG GXX CXG AAG GXG XXC XXG;

                                           (SEQ ID NO: 11)
k)   CAA CXG XXG CCX CCG GXX CXG AAG GXG XXC;
and

                                           (SEQ ID NO: 12)
l)   XXG CCX CCG GXX CXG AAG GXG XXC XXG XAC,
```

wherein X=U or T, wherein the oligomer's base sequence can vary from the above sequence at up to two base positions, and

wherein the molecule can bind to a target site to cause exon skipping in an exon of the dystrophin gene.

2. The oligomer according to claim **1**, wherein the exon of the dystrophin gene at which exon skipping is caused is selected from the group consisting of exon 44, 45, 46, and 53.

3. The oligomer according to claim **1**, wherein the oligomer causes an exon skipping rate of at least 50%.

4. The oligomer according to claim **1**, wherein the oligomer is between 25 and 35 bases in length.

5. The oligomer according to claim **1**, wherein the oligomer is 30 bases in length.

6. The oligomer according to claim **1**, wherein the oligomer is conjugated to or complexed with a distinct chemical entity.

7. The oligomer according to claim **1**, wherein the oligomer is a phosphorodiamidate morpholino oligonucleotide (PMO).

8. A vector for ameliorating DMD, the vector encoding an oligomer according to claim **1**, wherein when introduced into a human cell the oligomer is expressed.

9. A pharmaceutical composition for ameliorating DMD, the composition comprising an oligomer according to claim **1** or a vector according to claim **8**, and a pharmaceutically acceptable carrier, adjuvant or vehicle.

10. A pharmaceutical composition according to claim **9** comprising a plurality of oligomers or vectors encoding oligomers, or a combination of the oligomers and vectors, wherein the oligomers and/or vectors in the pharmaceutical composition cause skipping in a plurality of exons.

11. A method of ameliorating DMD in a human patient, the method comprising: administering a therapeutically effective amount of an oligomer according to claim **1** or a vector according to claim **8** to the patient such that the DMD is ameliorated.

12. The method according to claim **11** comprising administering a therapeutically effective amount of a plurality of oligomers to the patient, wherein the oligomers cause skipping in a plurality of exons.

* * * * *

EXHIBITS AY-BL REDACTED IN THEIR ENTIRETY

EXHIBIT BM

REDACTED

International Journal of Toxicology OnlineFirst, published on January 28, 2010 as doi:10.1177/1091581809359206

International Journal of Toxicology
000(00) 1-14
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DOI: 10.1177/1091581809359206
http://ijt.sagepub.com
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Safety Pharmacology and Genotoxicity Evaluation of AVI-4658

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Abstract

Duchenne muscular dystrophy (DMD) is caused by dystrophin gene mutations. Restoration of dystrophin by exon skipping was demonstrated with the phosphorodiamidate morpholino oligomers (PMO) class of splice-switching oligomers, in both mouse and dog disease models. The authors report the results of Good Laboratory Practice–compliant safety pharmacology and genotoxicity evaluations of AVI-4658, a PMO under clinical evaluation for DMD. In cynomolgus monkeys, no test article–related effects were seen on cardiovascular, respiratory, global neurological, renal, or liver parameters at the maximum feasible dose (320 mg/kg). Genotoxicity battery showed that AVI-4658 has no genotoxic potential at up to 5000 μg/mL in an in vitro mammalian chromosome aberration test and a bacterial reverse mutation assay. In the mouse bone marrow erythrocyte micronucleus test, a single intravenous injection up to 2000 mg/kg was generally well tolerated and resulted in no mutagenic potential. These results allowed initiation of systemic clinical trials in DMD patients in the United Kingdom.

Keywords

Exon skipping, phosphorodiamidate morpholino oligomer, genotoxicity, antisense oligonucleotide, safety pharmacology

Duchenne muscular dystrophy (DMD) affects 1 in every 3500 male and, in rare cases, female newborns worldwide[1] and results from mutations of the dystrophin gene. Lack of dystrophin leads to reduced sarcolemmal stability with actin filament contraction and increased intracellular calcium influx followed by muscle fiber degeneration. The clinical effect of a disrupted reading frame in the dystrophin gene is dramatic and lethal.[1,2] In DMD patients, the first symptoms involve the lower limbs and appear between the third and fifth year. These boys develop hypertrophic calves, show difficulty in running and climbing stairs, run on their tiptoes, and frequently fall. Muscle weakness progresses to the shoulder girdle upper arm and trunk muscles and loss of ambulation before the age of 12 years. Histological changes are readily apparent with light microscopy analysis of cross sections from patient muscle biopsies. They involve variation in fiber size with atrophic and hypertrophic fibers, degeneration and regeneration of the muscle fibers, infiltration of inflammatory cells and fibrosis, and characteristic central location of the nuclei within muscle cells. The fiber membrane destabilization results in leakage of the enzyme creatine kinase (CK), resulting in very high serum CK levels (20 000 to 50 000 U/L compared with 80 to 250 U/L in unaffected individuals). These levels decline as the patients get older, and the overall muscle mass decreases progressively. One-third of all affected boys are mentally impaired, and learning difficulties are common. Due primarily to the loss of muscle strength and integrity, DMD patients usually die in their 20s from cardiorespiratory failure.[1,2]

Despite extensive effort, no effective disease-modifying therapy for DMD is yet available. However, a delay of the onset of disease manifestations and an improvement in quality of life can be achieved by drugs that decelerate progression of the DMD pathology. These include glucocorticoids,[3] most commonly prednisolone and deflazocort, which can improve muscle strength and delay loss of ambulation by up to 2 to 3 years.[1] The mechanisms of their beneficial effect are not well understood but likely include anti-inflammatory activity, which may prevent the additional damage caused by the infiltration of mononuclear cells into the muscle upon necrosis.[4] However, the benefits of glucocorticoid therapy come at a price of frequent side effects, which can include obesity, spine deformities, bone loss, and growth retardation.[5,6] To date, the most effective treatment for prolonging the life of DMD boys has been assisted ventilation with portable ventilators. Ventilation has been shown to increase the average life expectancy of DMD boys from 19 to 24 years. All of the above treatments are palliative and do not address the underlying cause of the disease: loss of dystrophin expression. No current treatment reverses or arrests the progression of DMD.

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In addition to DMD, a milder, allelic form of muscular dystrophy called Becker muscular dystrophy (BMD) exists.[1,2] In BMD, unlike DMD, the reading frame is not disrupted, and an internally truncated yet functional dystrophin protein is produced. Most BMD patients remain ambulant for life and have a near-normal life expectancy. Work by Kole and others proposed the use of antisense oligonucleotides to modulate dystrophin mRNA splicing and convert out-of-frame DMD mutations into the nearest in-frame BMD-like mutation, to produce an internally deleted Becker-like functional dystrophin protein.[7-10] The mechanism of splice-switching oligomer (SSO) modulation of dystrophin pre-mRNA splicing involves hybridization to specific motifs involved in splicing and exon recognition in the pre-mRNA. This prevents normal spliceosome assembly and results in skipping of the target exon in the mature RNA transcript.[11,12] In the case of out-of-frame dystrophin gene deletions, selective removal of specific flanking exons should result in in-frame mRNA transcripts that may be translated into an internally deleted, BMD-like, and functionally active dystrophin protein.[9,10]

Unmodified DNA and RNA oligonucleotides have poor in vivo stability and therefore are ineffective as drugs. The most common chemical modification used in early-generation oligonucleotides to improve the stability and pharmacokinetics was the introduction of the phosphorothioate linkage in place of the natural phosphodiester linkage. These phosphorothioate compounds have been used extensively in both preclinical and clinical evaluations, and the dose-limiting toxicities and adverse effects are well established.[13] Initial evaluations in primates led to mortality following intravenous bolus injections of phosphorothioate oligonucleotides at doses as low as 10 mg/kg,[14] while other effects included lethargy, central hypotension, and reduced cardiac output. Other notable toxic effects associated with phosphorothioate oligonucleotides include complement activation and prolonged coagulation times.[15] The latter, specifically linked to the phosphorothioate component of the oligonucleotides, is therefore considered a class effect,[16] as were observed hepatotoxic effects.[17] These dose-limiting toxicities are most likely due to high C_{max} achieved with bolus injections. Studies subsequent to those in which mortality was observed have typically been limited to lower doses and increased infusion times to reduce the toxic effects.[13]

To be effective in modulating splicing, SSOs must not activate RNA cleavage by RNase H, which would destroy the pre-mRNA before splicing can occur.[18] Phosphorodiamidate morpholino oligomers (PMOs) productively compete with the splicing factors for target sequences in pre-mRNA during splicing, and in addition, their stability and in vivo uptake and bioavailability are improved compared with natural oligonucleotides. PMOs have standard nucleic acid bases attached to the morpholino-phosphoroamidate backbone (Figure 1), which, unlike other sugar-phosphate backbone oligonucleotides, is uncharged.[19] PMOs are very resistant to enzymatic degradation in vivo, providing unparalleled stability and somewhat different biodistribution than other oligonucleotides. Other modifications that can be used as SSOs include previous-generation chemistries such as the 2′-O–substituted 2′-O-methyl phosphorothioate (2′OMe), and locked nucleic acids/phosphorothioate.[20]

AVI-4658 is a PMO drug with the general structure described in Figure 1, with the base sequence CTC CAA CAT CAA GGA AGA TGG CAT TTC TAG. It is designed to skip exon 51 of human dystrophin and thus restore dystrophin expression in DMD patients having certain mutations.[21] AVI-4658 targets the pre-mRNA transcripts of the dystrophin gene, causing exon 51 to be skipped from the mature, spliced mRNA. In cells from DMD patients with deletions in exons 50, 52, 52-63, 45-50, 48-50, or 49-50, exon skipping restored or is expected to restore the reading frame and produce an internally truncated, BMD-like form of dystrophin. Here, we report the results of a safety pharmacology evaluation of the PMO AVI-4658 in cynomolgus monkeys following intravenous and subcutaneous administration at doses up to the maximum feasible dose of 320 mg/kg. We also report the results of a standard genotoxicity battery evaluation using AVI-4658 at concentrations up to 5000 µg/mL in an in vitro mammalian cell chromosome aberrations test, up to 5000 µg/plate in a bacterial reverse mutation assay, and up to 2000 mk/kg as a single intravenous administration in a mouse micronucleus assay.

Materials and Methods

The safety pharmacology evaluation was performed by MDS Pharma Services (Lyon, France). The testing facility is Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited, and the study plan was reviewed by the ethical committee, according to the following animal health and welfare guidelines: guide for the care and use of laboratory animals, NRC, 1996, Decree no. 2001-464 regarding the experiments with laboratory animals described in the *Journal Officiel de la République Française* on May 29, 2001, Decree no. 2001-486 relating to the protection of animals used in scientific experiments described in the *Journal Officiel de la République Française* on June 6, 2001. The study was conducted according to the following: guideline on safety pharmacology studies for human pharmaceuticals (November 8, 2000, issued as CPMP/ICH/539/00–ICH S7A, published in the *Federal Register*, vol 66, no. 135, July 13, 2001, pp 36791-36792) and guideline on nonclinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals (May 12, 2005, issued as CPMP/ICH/423/02–ICH S7B, published in the *Federal Register*, vol 70, no. 202, October 20, 2005, pp 61133-61134). All phases of this study performed at the testing facility were conducted in compliance with the following Good Laboratory Practice (GLP) regulations: OECD *Principles of Good Laboratory Practice* concerning mutual acceptance of data in the assessment of chemicals, dated November 26, 1997, (C[97] 186 Final), "Principles of Good Laboratory Practice" described in the *French Official Journal* on March 23, 2000, Organization for Economic Co-operation and Development (OECD) GLP consensus document (the application of the

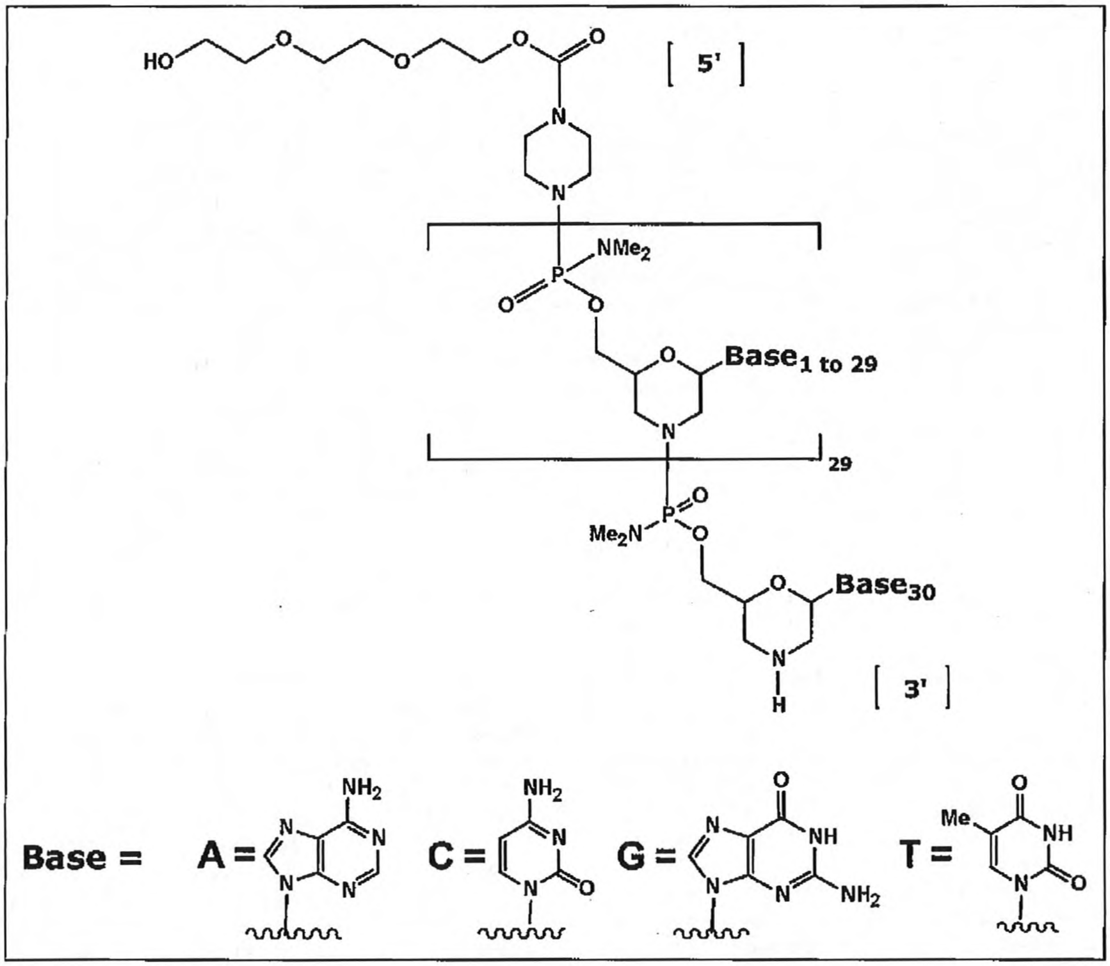

Figure 1. Structure of phosphorodiamidate morpholino oligomers.

OECD principles of GLP to the organization and management of multisites studies, ENV/JM/MONO [2002]9, June 25, 2002).

The genotoxicity battery was performed by BioReliance (Rockville, MD). This study was conducted in compliance with the most recent version of the US Food and Drug Administration GLP regulations, 21 CFR part 58, and the OECD *Principles of Good Laboratory Practice*, C(97)186/Final, and in compliance with the testing guidelines ICH S2A (1996), ICH S2B (1997), and OECD 474 (1998). The number of mice and the procedures and experimental design used for this study have been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee 8 and 10. All procedures involving mice performed at BioReliance follow the specifications recommended in *The Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC, 1996). The mice were housed in an AAALAC-accredited facility.

Safety Pharmacology Evaluation of AVI-4658

Animals and animal husbandry. Six male cynomolgus monkeys (*Macaca fascicularis*) were used in this study, with a weight range of 2.7 to 2.9 kg and an age range of 2 to 3 years. Animals were housed in 1 room for the study in an air-conditioned building with a target temperature of 22°C ± 2°C, relative humidity >40%, a minimum 10 air changes per hour, and 12 hours light (artificial)/12 hours dark. Animals were housed singly in stainless steel mesh cages. Animals were fed expanded complete commercial primate diet at approximately 100 g diet/animal per day. In addition, animals received fruit or vegetable daily (apple, banana, or carrot). Certificates of analysis for the diet and drinking water are maintained in the archives of the testing facility, which conducted the tests according to current animal welfare guidelines. The normal dark cycle was interrupted on occasions (for up to 45 minutes)

Table 1. Study Design

Treatment Group	Dose Level, mg/kg	Number of Animals	Dose Volume, mL/kg
Phase 1, subcutaneous dosing			
Vehicle (PBS)	0	6	2.67
Low dose	40	6	2.67
Intermediate dose	160	6	2.67
High dose	320	6	2.67
Phase 2, intravenous dosing			
Vehicle (PBS)	0	6	2.67
High dose	320	6	2.67

Abbreviation: PBS, phosphate-buffered saline.

to allow completion of technical procedures. These differences were not considered to have affected the outcome of the study.

Procedures for telemetry and venous catheter implantation. Animals were fasted for at least 15 hours before surgical procedures. For implantation of the telemetry device, each animal was premedicated with a subcutaneous injection of glycopyrrolate (Robinul V, Vétoquinol SA; 0.01 mg/kg) and then anesthetized with an intramuscular injection of ketamine (Imalgène 500, Mérial SAS; 15 mg/kg) and xylazine hydrochloride (Rompun 2%, Bayer AG; 0.7 mg/kg). In addition, local oropharyngeal anesthesia was provided with a spray of lidocaine chlorhydrate (Xylocaine 5 % Nébuliseur, AstraZeneca). The hair on the abdomen, the inguinal area, and the thorax was clipped. During surgery, the level of anesthesia was maintained with gaseous anesthetic (1% to 5% isoflurane in oxygen, AErrane, Laboratoire Baxter). An antibiotic prophylaxis by intramuscular injection with long-acting amoxycilline (Clamoxyl LA, Pfizer Italia SRL) at 30 mg/kg was given 48 hours before surgery. The transmitter body was implanted into the abdominal cavity under aseptic conditions. The pressure catheter (polyurethane tubing that extends out of the device body) was inserted into the lower abdominal aorta via the femoral artery and the bipotential leads then placed. Animals received antibiotic prophylaxis by intramuscular injection with long-acting amoxycilline (Clamoxyl LA, Pfizer; 30 mg/kg) and an analgesic prophylaxis intramuscular injection of tolfenamic acid (4% Tolfédine, Vétoquinol; 4 mg/kg), once right after implantation and then repeated 4 times at 48-hour intervals. The surgical wounds were disinfected with iodine (Vétédine, Vétoquinol) for 7 days. After surgery, animals were allowed to recover for approximately 3 weeks before the first treatment. Prior to intravenous dosing, the animals were implanted with a venous catheter, under anesthesia similar to that above. The catheter was attached to the delivery system via a tether and a swivel joint.

Safety examinations. Arterial blood pressure, heart rate, electrocardiogram, respiratory parameters, global neurological activity, and renal and liver functions were examined following 3 separate subcutaneous administrations and 1 single intravenous administration of AVI-4658 in the conscious male cynomolgus monkey. All observations, including respiratory parameters, were collected from the freely moving animal. AVI-4658 was administered by the subcutaneous route (phase 1) at 0 (vehicle), 40, 160, and 320 mg/kg on days 0, 7, 14, and 21 and by the intravenous route (phase 2) at 0 (vehicle) and 320 mg/kg on days 38 and 45 using a dosing volume of 2.67 mL/kg with an infusion rate of 1 mL/min (Table 1). Subcutaneous and intravenous dosing were examined to support both routes for potential clinical administration. The high dose (320 mg/kg) was used to support up to 100 mg/kg clinically, based on allometric scaling. For intravenous dosing, 320 mg/kg was the maximum feasible dose based on dose volume and solubility of the compound. The low dose for phase 1 was selected based on the expected therapeutic dose (human equivalent dose of approximately 10 mg/kg). For phase 1, animals were randomized in a Latin square design. For both phases, there were at least 6 days between each testing session. Each animal served as its own control.

Telemetry signals (body temperature, cardiovascular and respiratory parameters) were recorded. Time points were selected to correspond to the times of maximum exposure to the drug:

- phase 1: on days 0, 7, 14, and 21, starting at least 1.5 hours preadministration and for 21 hours postadministration
- phase 2: on days 38 and 45, starting at least 1.5 hours preadministration and for 24 hours postadministration

The telemetric system used consisted of an implantable TL11M3-D70-CCTP device (DSI, St Paul, MN), an RMC-1 receiver located on the top of each cage, a DEM data exchange matrix that centralizes the signals from all animals, an APR-1 ambient pressure reference that allows a barometric correction, and a microcomputer with acquisition card. For collecting and analyzing hemodynamic, cardiac, and respiratory parameters, Notocord-hem software (Notocord Systems SA, Croissy-sur-Seine, France) was used.

For cardiovascular analyses, the value for each parameter (systolic blood pressure, mean arterial blood pressure, diastolic blood pressure, and heart rate) was the mean of the values recorded for 5 minutes around the time point (selected times $\pm$2.5 minutes). The value for each interval or complex (PR, RR, QT, or QRS) was the mean of the 10 best quality electrocardiogram (ECG) traces at the time point (selected time $\pm$2.5 minutes). The value for respiration rate (f), inspiratory time (TI), expiratory time (TE), and AUC_{EMG} was the mean of 10 values obtained around the time point (selected time $\pm$2.5 minutes). Note that the signals recorded at the predefined time points were occasionally disturbed. In this case, data recorded a few minutes before or a few minutes after the theoretical time point were used instead. On a few occasions, electromyogram (EMG) parameter values were calculated from fewer than 10 values. In another few cases, sustained disturbances of the signal did not allow an accurate evaluation of any data at the

predefined time point (detailed in the raw data). The results were expressed as mean ± standard error of the mean (SEM).

For respiration parameters, inspiratory time (TI, milliseconds) was defined as the duration of the diaphragmatic EMG burst. Expiratory time (TE, milliseconds) was defined as the time elapsed between 2 diaphragmatic EMG bursts, and respiration rate (f, breath/min) was calculated from the averaged respiratory cycle duration (TI + TE). The AUC of the rectified diaphragmatic EMG burst (AUC_{EMG}) is that for which variation in amplitude has been shown to be correlated in humans and animals with variation of the tidal volume.[22-24] For this evaluation, the raw EMG signal is filtered and rectified. Moreover, AUC_{EMG} is normalized such that AUC_{EMG} values are expressed as a percentage change from the pretest value.

For neurological evaluations, all animals were examined at pretest and approximately 4 hours and 8 hours after treatment. Evaluated parameters included level of consciousness, motor function, and eye movements. Normal versus abnormal results were recorded and graded for each major parameter at each time point. All results for all animals were normal and noted as follows: level of consciousness: −1 = alert (normal); motor function: −1 = normal; eye movements: −1 = normal fixation and following of stimulus. Hepatic function evaluation, hematology, and other clinical chemistry analyses were performed on day −4 and day 24. Renal function evaluation and urine analysis were performed on day −3 and day 25.

Genotoxicity Evaluation of AVI-4658

Bacterial reverse mutation assay. The tester strains used were the *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA1535, and TA1537 as described by Ames et al[25] and *Escherichia coli* WP2 *uvr*A as described by Green and Muriel.[26] *Salmonella* tester strains were received from Dr Bruce Ames' designated distributor, Discovery Partners International (San Diego, CA). The *E coli* tester strain was received from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). Overnight cultures were prepared by inoculating from the appropriate master plate or from the appropriate frozen permanent stock into a vessel containing ~50 mL of culture medium. To ensure that cultures were harvested in the late log phase, the length of incubation was controlled and monitored. Aroclor 1254-induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from male Sprague-Dawley rats induced with a single intraperitoneal injection of Aroclor 1254, 500 mg/kg, 5 days prior to sacrifice. The S9 was prepared by and purchased from MolTox (Boone, NC). Upon arrival at BioReliance, the S9 was stored at −60°C or colder until used. Each bulk preparation of S9 was assayed for its ability to metabolize at least 2 promutagens to forms mutagenic to *S typhimurium* TA100. The S9 mix was prepared immediately before its use and contained 10% S9, 5 mM glucose-6-phosphate, 4 mM β-nicotinamide-adenine dinucleotide phosphate, 8 mM $MgCl_2$, and 33 mM KCl in a 100 mM phosphate buffer at pH 7.4. The sham S9 mixture (sham mix), containing 100 mM phosphate buffer at pH 7.4, was prepared immediately before its use. To confirm the sterility of the S9 and sham mixes, a 0.5-mL aliquot of each was plated on selective agar.

In the initial toxicity-mutation assay, the maximum dose of AVI-4658 tested was 5000 µg per plate; this dose was achieved using a concentration of 50 mg/mL and a 100-µL plating aliquot. The dose levels tested were 1.5, 5.0, 15, 50, 150, 500, 1500, and 5000 µg per plate. The test article formed soluble and clear solutions in sterile water for injection from 0.015 to 50 mg/mL. Neither precipitate nor background lawn toxicity was observed. No positive mutagenic responses were observed with any of the tester strains in either the presence or absence of S9 activation.

Based on the findings of this initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate. For the confirmatory mutagenicity assay, the dose levels tested were 50, 150, 500, 1500, and 5000 µg per plate.

On the day of its use, minimal top agar, containing 0.8 % agar (W/V) and 0.5 % NaCl (W/V), was melted and supplemented with L-histidine, D-biotin, and L-tryptophan solution to a final concentration of 50 µM each. Top agar not used with S9 or sham mix was supplemented with 25 mL of water for each 100 mL of minimal top agar. For the preparation of media and reagents, all references to water imply sterile, deionized water produced by the Milli-Q Reagent Water System. Bottom agar was Vogel-Bonner minimal medium E (Vogel and Bonner, 1956) containing 1.5 % (W/V) agar. Nutrient bottom agar was Vogel-Bonner minimal medium E containing 1.5 % (W/V) agar and supplemented with 2.5 % (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Nutrient Broth was Vogel-Bonner salt solution supplemented with 2.5% (W/V) Oxoid Nutrient Broth No. 2 (dry powder). Each plate was labeled with a code system that identified the test article, test phase, dose level, tester strain, and activation, as described in detail in BioReliance's *Standard Operating Procedures*. One-half (0.5) milliliter of S9 or sham mix, 100 µL of tester strain, and 100 µL of vehicle or test article dilution were added to 2.0 mL of molten selective top agar at 45°C ± 2°C. After vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar. When plating the positive controls, the test article aliquot was replaced by a 50-µL aliquot of appropriate positive control. After the overlay had solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37°C ± 2°C. Plates that were not counted immediately following the incubation period were stored at 2°C to 8°C until colony counting could be conducted.

The condition of the bacterial background lawn was evaluated for evidence of test article toxicity by using a dissecting microscope. Precipitate was evaluated after the incubation period by visual examination without magnification.

Revertant colonies for a given tester strain and activation condition, except for positive controls, were counted either entirely by automated colony counter or entirely by hand unless the plate-exhibited toxicity.

Table 2. Mouse Bone Marrow Micronucleus Test Design

Treatment	Number of Mice/Sex Dosed	Number of Mice/Sex	
		24 Hours Postdose	48 Hours Postdose
Phosphate-buffered saline (20 mL/kg)	10	5	5
Low dose: 500 mg/kg (100 mg/mL × 5 mL/kg)	5	5	—
Mid dose: 1000 mg/kg (100 mg/mL × 10 mL/kg)	5	5	—
High dose: 2000 mg/kg (100 mg/mL × 20 mL/kg)	10	5	5
Positive control: CP 50 mg/kg (2.5 mg/mL × 20 mL/kg)	5	5	—

In vitro mammalian chromosome aberration test. Chinese hamster ovary (CHO-K1) cells (repository no. CCL 61) were obtained from American Type Culture Collection (Manassas, VA). The use of CHO cells has been demonstrated to be an effective method of detection of chemical clastogens.[27] Aroclor 1254–induced rat liver S9 was used as the metabolic activation system as above. The S9 was thawed and mixed with a cofactor pool to contain 2 mM magnesium chloride, 6 mM potassium chloride, 1 mM glucose-6-phosphate, 1 mM nicotinamide adenine dinucleotide phosphate, and 20 µL S9 per milliliter medium (McCoy's 5A serum-free medium supplemented with 100 U penicillin/mL, 100 µg streptomycin/mL, 2 mM L-glutamine, and 2.5 µg/mL amphotericin B).

A preliminary toxicity assay was performed for the purpose of selecting dose levels for the chromosome aberration assay and consisted of an evaluation of test article effect on cell growth.[28] CHO cells were seeded at approximately 5×10^5 cells/25 cm^2 flask and were incubated (37°C ± 1°C in a humidified atmosphere of 5% ± 1% CO_2 in air). Treatment was carried out by refeeding the flasks with 4.5 mL complete medium (McCoy's 5A medium supplemented with 10% fetal bovine serum [FBS], 100 U penicillin/mL, 100 µg streptomycin/mL, 2 mM L-glutamine, and 2.5 µg/mL amphotericin B) for the nonactivated study or S9 reaction mixture (3.5 mL serum-free medium plus 1 mL of S9/cofactor pool) for the activated study, to which was added 500 µL dosing solution of test article in vehicle or vehicle alone. Therefore, the final concentrations of serum-free medium components were diluted by 30% and S9 cofactor pool by 80%. The cells were exposed to vehicle alone and to 1 of 9 final concentrations (0.5 to 5000 µg/mL) of the test article for 4 hours in both the presence and absence of S9 activation and for 20 hours continuously in the absence of S9 activation. After the 4-hour exposure period, the treatment medium was removed, the cells washed with calcium and magnesium-free phosphate buffered saline (CMF-PBS), and refed with 5 mL complete medium. At 20 hours after the initiation of treatment, the cells were harvested by trypsinization and counted using a Coulter counter. Cell viability was determined by trypan blue dye exclusion.

The chromosome aberration assay was performed using standard procedures.[29] The CHO cells were seeded and treated as above. In the absence of both test article precipitation in the treatment medium and at least 50% toxicity, the highest dose level evaluated was 5000 µg/mL. After the exposure period, the treatment medium was removed, the cells washed with CMF-PBS, and refed with complete medium. Two hours prior to the scheduled cell harvest, Colcemid was added to duplicate flasks for each treatment condition at a final concentration of 0.1 µg/mL, and the flasks were returned to the incubator until cell collection. A concurrent toxicity test was conducted in both the nonactivated and the S9-activated test systems. Two hours after the addition of Colcemid, metaphase cells were harvested for both the nonactivated and S9-activated studies by trypsinization. Cells were collected and chromosome sample slides prepared and analyzed as described previously.[30] Slides were coded using random numbers by an individual not involved with the scoring process. Mitomycin C was used as the positive control in the nonactivated study at final concentrations of 0.1 and 0.2 µg/mL. Cyclophosphamide was used as the positive control in the S9-activated study at final concentrations of 10 and 20 µg/mL.

Mouse bone marrow micronucleus test. ICR mice were obtained from Harlan (Frederick, MD). At the time of use, the mice were 6 to 8 weeks old.

The mice were observed each day for signs of illness and other conditions of poor health. All mice were judged to be healthy prior to utilization in the study.

The micronucleus study was conducted using established and validated procedures.[31] Following an initial dose-range–finding study to determine the high dose, mice in the definitive micronucleus study were assigned to 7 experimental groups (5 of which were killed at 24 hours and 2 groups that were extended to 48 hours) of 5 males and 5 females each. The study design is shown in Table 2.

AVI-4658 was administered at a dose volume of up to 20 mL/kg by a single intravenous injection into the lateral tail vein by using 1 mL disposable polypropylene syringes with hypodermic needles (27G). At the scheduled bone marrow collection time, 5 mice per sex per treatment were euthanized by CO_2 asphyxiation. Immediately following euthanasia, the femurs were exposed, cut just above the knee, and the bone marrow was aspirated into a syringe containing FBS. The bone marrow cells were transferred to a labeled centrifuge tube containing approximately 1 mL FBS. The tubes were identified by labels containing the study, group, and animal numbers. The bone marrow cells were pelleted by centrifugation at approximately 100*g* for 5 minutes, and the supernatant was drawn off, leaving a small amount of serum with the remaining cell pellet. The cells were resuspended, and a small drop of bone marrow suspension was spread onto a clean glass slide. Two slides were prepared from each mouse and fixed in methanol. One set of slides was stained with May-Gruenwald-Giemsa stain and permanently mounted and used in microscopic evaluation. Using a light microscope and a medium magnification (400×), an area

of acceptable quality was selected such that the cells were well spread and stained.

Using oil immersion (1000×), the following cell populations and cellular components were evaluated and enumerated: polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs), and micronuclei. Two thousand PCEs per mouse were scored for the presence of micronuclei, resulting in evaluation of a total of 10 000 PCEs per each treatment group. To control for bias, bone marrow slides were coded using a random number table by an individual not involved with the scoring process. The number of NCEs and micronucleated NCEs (MNCEs) in the field of 1000 total erythrocytes (ECs) was determined for each animal to determine the proportion of polychromatic erythrocytes to total erythrocytes (PCEs/ECs). Micronuclei are round, darkly staining nuclear (chromosome) fragments with a sharp contour and diameters usually from 1/20 to 1/5 of an erythrocyte. Micronuclei may occur in PCEs (MPCEs) or NCEs (MNCEs). The proportion of PCEs/ECs was also recorded per 1000 ECs per each animal.

Statistical significance was determined using the Kastenbaum-Bowman tables, which are based on the binomial distribution.[32] All analyses were performed separately for each sex and sampling time. To quantify the proliferation state of the bone marrow as an indicator of bone marrow toxicity, the proportion of PCEs/ECs was determined for each mouse and treatment group. The proportion of PCEs/ECs in test article–treated animals should not be less than 20% of the control value. The test article would have been considered to induce a positive response if a dose-responsive increase in the incidence of MPCEs is observed and 1 or more doses are statistically elevated relative to the vehicle control ($P < .05$, Kastenbaum-Bowman tables) at any sampling time.

Results

Safety Pharmacology Evaluation of AVI-4658

AVI-4658 administration did not affect the health status and body weights of animals throughout the study period. Furthermore, no injection site reactions were detected following any of the subcutaneous or intravenous administrations.

Various cardiovascular parameters were measured. AVI-4658 injected intravenously at a high dose of 320 mg/kg had no effect on either arterial, systolic, or diastolic blood pressure; heart rate, QT and QTc interval durations (Figure 2); or RR and PR intervals and QRS complex durations (data not shown). Similarly, subcutaneous administration had no effect on any of these parameters (data not shown). These data show that single, high-dose administrations of the AVI-4658 PMO compound do not alter key heart function parameters.

Intravenous and subcutaneous injections had no effect on the respiratory rate, inspiratory rate, expiratory rate, or AUC_{EMG} (Figure 3; subcutaneous data not shown); AUC_{EMG} is a marker of tidal volume compared with the pretest values. These data demonstrated the pulmonary safety of AVI-4658, even at high doses. No neurological changes, including level of consciousness, motor function, and eye movements, were detected following any dose administration of AVI-4658.

In addition to the evaluations listed above, the cynomolgus monkeys were evaluated for markers of renal and hepatic function; a panel of urinalysis, hematology, and serum chemistry tests were performed prior to and following the subcutaneous administration phase. The data show that AVI-4658 administration did not affect blood urea nitrogen, urine or serum creatinine, alanine aminotransferase, aspartate aminotranferase, and serum albumin (Figure 4A-D). The glomerular filtration rate and free water clearance were not affected (Figure 4E), nor were key hematological parameters, including activated partial thromboplastin time and prothrombin time, affected (Figure 4F). Additional urine, hematology, and serum chemistry parameters also showed no alterations as a result of AVI-4658 administration.

Genotoxicity Evaluation of AVI-4658

Bacterial reverse mutation assay. AVI-4658 was tested in the bacterial reverse mutation assay using *S typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *E coli* tester strain WP2 *uvr*A in the presence and absence of Aroclor-induced rat liver S9. In the initial toxicity-mutation assay, doses from 1.5, 5.0, 15, 50, 150, 500, 1500, and 5000 µg per plate were tested. In the initial toxicity-mutation assay, no positive mutagenic response, precipitate, or background lawn toxicity were observed (data not shown). Based on the findings of the initial toxicity-mutation assay, the maximum dose plated in the confirmatory mutagenicity assay was 5000 µg per plate. In this assay, no positive mutagenic response was observed at the dose levels tested of 50, 150, 500, 1500, and 5000 µg per plate. For all test strains, the numbers of revertant colonies on plates treated with AVI-4658 at any dose were similar to those in the vehicle control, both in the presence and absence of metabolic activation by rat liver S9 (Table 3). The positive control used in each tester strain induced significantly higher numbers of revertants than either the vehicle control or any of the AVI-4658–treated groups. Neither precipitate nor appreciable toxicity was observed at any dose level.

Data sets for tester strains TA1535/TA1537 and TA98/TA100/WP2 uvrA were judged positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 3.0 or 2.0 times the mean vehicle control value, respectively. In our tests, only the respective positive control treatments met the criteria for a positive increase in mean revertants compared with historical control data.

In vitro mammalian chromosome aberration test. AVI-4658 was tested in the chromosome aberration assay using CHO cells in both the absence and presence of an Aroclor-induced S9 activation system. Cytotoxicity was determined by the population doubling method. In the preliminary toxicity assay, the maximum dose tested was 5000 µg/mL, and no substantial toxicity (ie, at least 50% cell growth inhibition, relative to the vehicle control) was observed at any dose level (not shown).

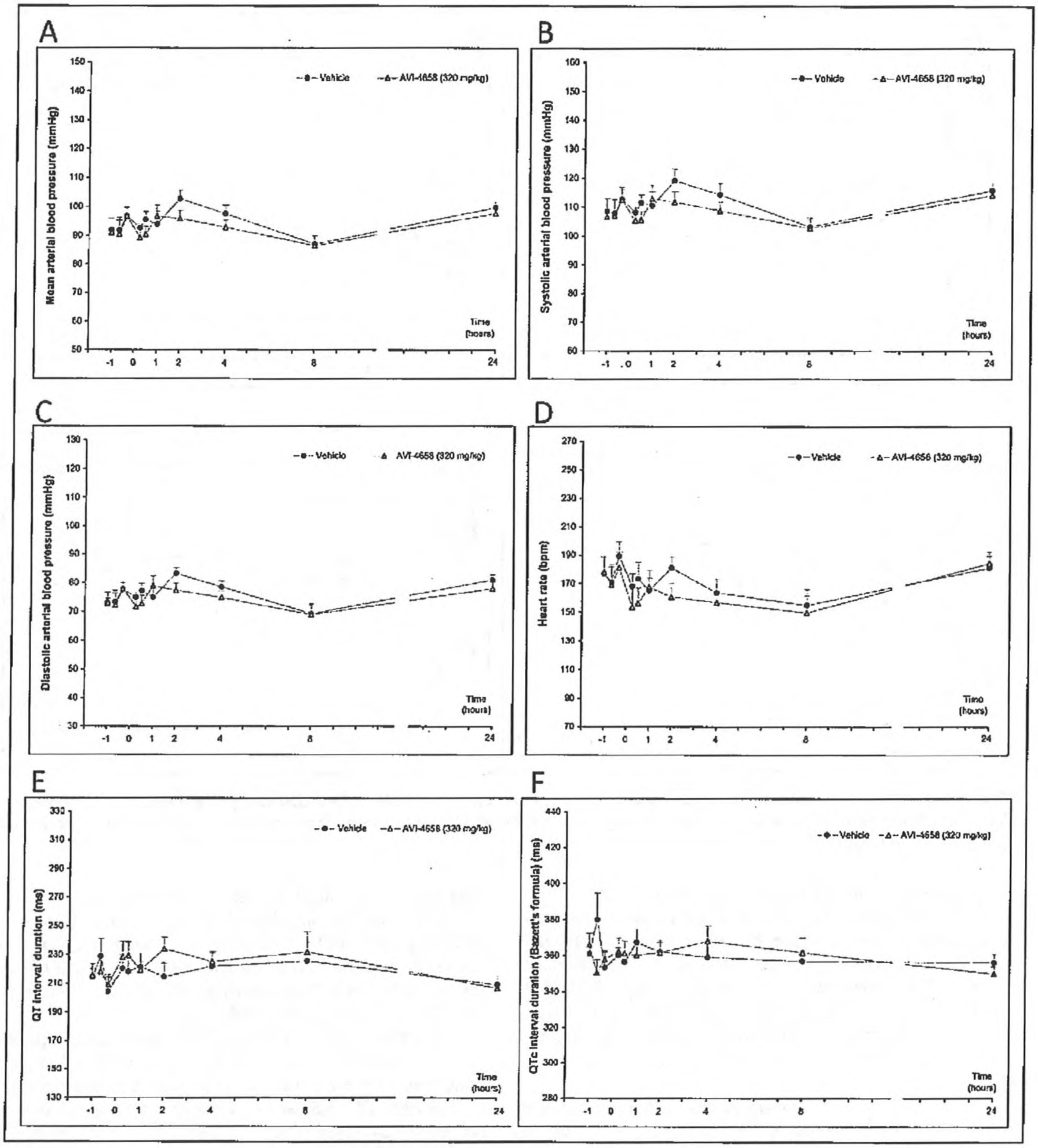

Figure 2. Evaluation of cardiac parameters following intravenous injections of AVI-4658 at 320 mg/kg or placebo in same animals. Parameters were measured up to 24 hours following injection, including (A) mean arterial blood pressure, (B) systolic blood pressure, (C) diastolic blood pressure, (D) heart rate, (E) QT interval, and (F) QTc interval.

Based on these findings, the doses chosen for the chromosome aberration assay ranged from 1250 to 5000 µg/mL for all 3 treatment conditions.

In the chromosome aberration assay, the cells were treated for 4 and 20 hours in the nonactivated test system and for 4 hours in the S9-activated test system. All cells were harvested

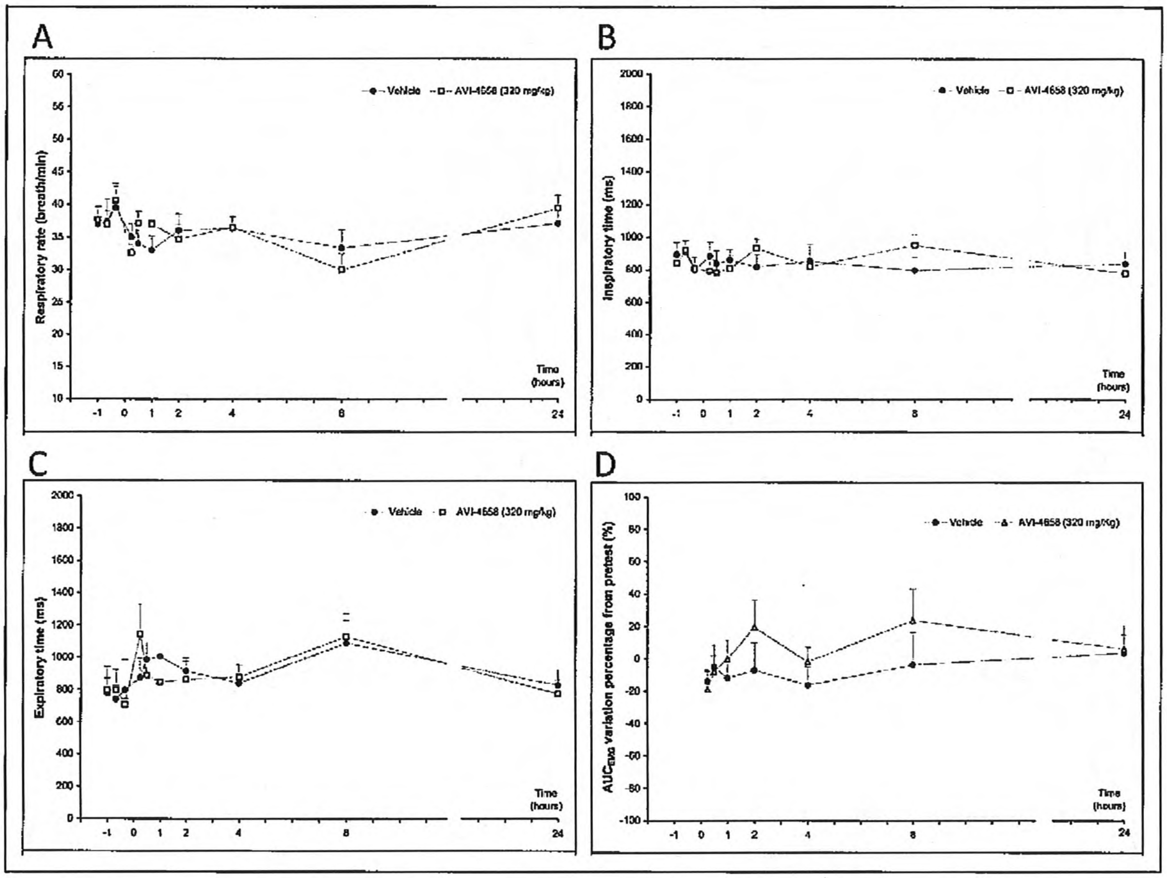

Figure 3. Evaluation of respiratory parameters following intravenous injections of AVI-4658 at 320 mg/kg or placebo in same animals. Parameters were measured up to 24 hours following injection, including (A) respiratory rate, (B) inspiratory rate, (C) expiratory time, and (D) AUC_{EMG} (a marker of tidal volume).

20 hours after treatment initiation. The results (Table 4) show that the percentage of cells with structural or numerical aberrations in the test article–treated groups was not significantly increased relative to vehicle control at any dose level ($P > .05$, Fisher exact test). Based on the findings of this study, AVI-4658 was concluded to be negative for the induction of structural and numerical chromosome aberrations in CHO cells in both nonactivated and S9-activated test systems.

Mouse bone marrow micronucleus test. The mouse micronucleus assay was performed in 2 phases. In the first phase, the dose-range–finding study was designed to investigate the toxicity of the test article and to set dose levels for the definitive micronucleus study. The second phase, the definitive micronucleus study, was designed to evaluate the potential of the test article to increase the incidence of micronucleated polychromatic erythrocytes in the bone marrow of male and female ICR mice.

In the dose-range–finding study, groups of 5 male and 5 female mice were exposed to AVI-4658 up to 2000 mg/kg intravenously. The highest dose was achieved by administration of a stock formulation (100 mg/mL) at a volume of 20 mL/kg. No mortality occurred at 2000 mg/kg, and only piloerection was noted in all the animals within an hour of dose administration. In the absence of mortality, the highest dose for the definitive micronucleus study was set at 2000 mg/kg.

The definitive micronucleus study consisted of 7 groups, each containing 5 male and 5 female mice. Mice in 5 of these groups were treated either with the controls (vehicle or positive) or with AVI-4658 at 500, 1000, or 2000 mg/kg and were euthanized 24 hours after treatment (see the Materials and Methods section). The remaining 2 groups were dosed with either control or 2000 mg/kg and euthanized 48 hours after treatment. At the time of euthanasia, femoral bone marrow was collected, and bone marrow smears (slides) were prepared and stained with May-Gruenwald-Giemsa stain. Bone marrow cells (PCEs) were examined microscopically for the presence of micronuclei (MPCEs). A statistical analysis of data was performed using the Kastenbaum-Bowman tables

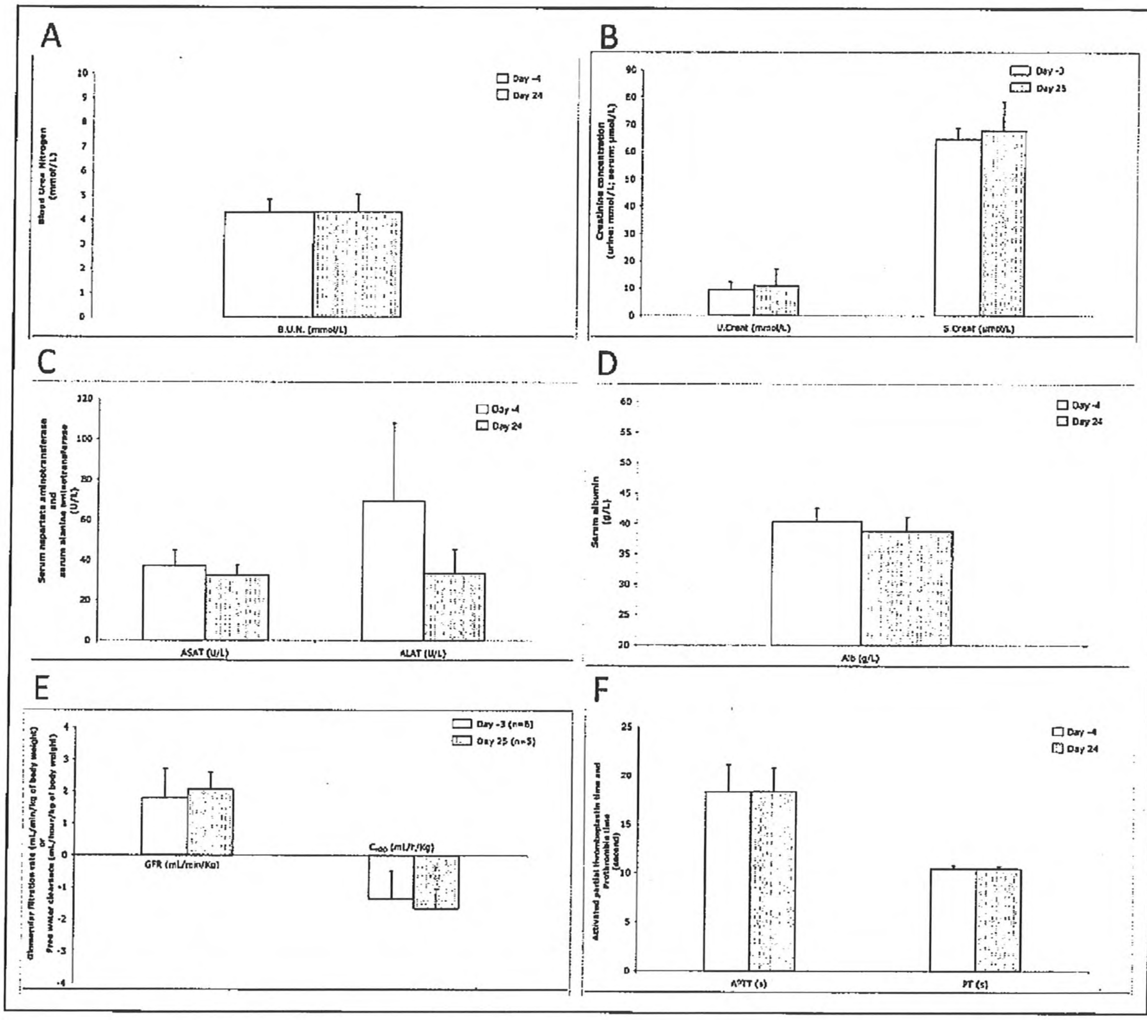

Figure 4. Evaluation of hepatic and renal function as well as serum chemistry and hematology following subcutaneous injection of AVI-4658 up to 320 mg/kg. Parameters were measured up to 24 hours following injection, including (A) blood urea nitrogen (BUN), (B) urine and serum creatinine, (C) aspartate aminotranferase (AST) and alanine aminotransferase (ALT), (D) serum albumin, (E) glomerular filtration rate (GFR) and water clearance, and (F) activated partial thromboplastin time (APTT) and prothrombin time (PT).

(binomial distribution, $P \leq .05$). The incidence of MPCEs and the ratio of PCEs/ECs served as indication of test article clastogenicity and cytotoxicity, respectively. The results are summarized in Table 5.

Slight reductions in the PCEs/ECs ratio, up to 13%, were observed in some of the AVI-4658 groups at 24 or 48 hours after dose administration relative to the vehicle control groups. However, the effects were not dose dependent, and reductions of this magnitude suggest that the test article did not inhibit erythropoiesis. No statistically significant increase in the incidence of MPCEs in the AVI-4658–treated groups was seen relative to the respective vehicle control groups in male or female mice at 24 or 48 hours after dose administration ($P > .05$, Kastenbaum-Bowman tables).

Cyclophosphamide, the positive control, induced a statistically significant increase in the incidence of MPCEs ($P \leq .05$, Kastenbaum-Bowman tables) in both male and female mice. The number of MPCEs in the vehicle control groups did not exceed the historical vehicle control range. Therefore, the controls used in this test were valid and showed that a single intravenous injection of AVI-4658 at doses up to and including 2000 mg/kg did not induce a significant increase in the incidence of MPCEs in bone marrow of male and female ICR mice.

Table 3. Bacterial Mutation Assay

Activation Condition: Dose, μg/plate	Average Revertants Per Plate ± SD TA98	TA100	TA1535	TA1537	WP2 *uvrA*
None					
Vehicle	21 ± 1	228 ± 9	19 ± 7	8 ± 3	24 ± 6
50	15 ± 4	213 ± 21	29 ± 6	10 ± 3	22 ± 4
150	14 ± 1	237 ± 9	25 ± 3	8 ± 3	18 ± 2
500	17 ± 5	228 ± 17	17 ± 6	11 ± 2	18 ± 3
1500	12 ± 2	227 ± 11	26 ± 9	7 ± 1	19 ± 6
5000	15 ± 4	209 ± 7	29 ± 5	7 ± 1	23 ± 2
Positive	610 ± 47	693 ± 31	450 ± 15	650 ± 103	132 ± 9
Rat liver S9 mix					
Vehicle	26 ± 4	212 ± 8	15 ± 6	8 ± 2	23 ± 2
50	28 ± 6	213 ± 11	19 ± 3	10 ± 2	23 ± 1
150	23 ± 3	235 ± 4	15 ± 3	10 ± 2	24 ± 3
500	17 ± 4	231 ± 18	15 ± 5	9 ± 2	24 ± 3
1500	18 ± 3	244 ± 9	16 ± 2	6 ± 3	24 ± 7
5000	17 ± 3	225 ± 17	15 ± 3	10 ± 4	22 ± 3
Positive	309 ± 14	711 ± 94	96 ± 14	93 ± 15	309 ± 12

Vehicle = vehicle control (sterile water for injection with or without S9 mix); Positive = positive control (S9 activation: all salmonella strains, 2-aminoantracene 1.0 μg/plate; WP2uvrA, 10 μg/plate); no activation = TA98, 1.0 μg/plate; TA100/TA1535, sodium azide, 1.0 μg/plate; TA1537, 9-aminoacridine, 75 μg/plate; WP2uvrA, methyl methanesulfonate, 1000 μg/plate).

Table 4. In Vitro Mammalian Chromosome Aberration Test

Treatment, μg/mL[a]	S9 Activation	Treatment Time	Mean Mitotic Index	Cells Scored: Numerical	Cells Scored: Structural	Aberrations per Cell, Mean ± SD[b]		Cells With Aberrations: Numerical, %	Cells With Aberrations: Structural, %
Water for injection	−S9	4	8.6	200	200	0.000	±0.000	0.0	0.0
AVI-4658									
1250	−S9	4	8.5	200	200	0.000	±0.000	0.5	0.0
2500	−S9	4	8.4	200	200	0.005	±0.071	0.5	0.5
5000	−S9	4	7.9	200	200	0.010	±0.141	0.0	0.5
MMC, 0.2	−S9	4	6.1	200	100	0.380	±0.826	0.0	23.0[c]
Water for injection	+S9	4	9.0	200	200	0.000	±0.000	1.0	0.0
AVI-4658									
1250	+S9	4	8.5	200	200	0.000	±0.000	1.0	0.0
2500	+S9	4	9.0	200	200	0.005	±0.071	0.5	0.5
5000	+S9	4	8.8	200	200	0.030	±0.264	1.0	1.5
CP, 10	+S9	4	6.9	200	100	0.420	±0.781	0.0	27.0[c]
Water for injection	−S9	20	9.6	200	200	0.000	±0.000	1.0	0.0
AVI-4658									
1250	−S9	20	9.4	200	200	0.010	±0.141	0.5	0.5
2500	−S9	20	8.6	200	200	0.005	±0.071	0.0	0.5
5000	−S9	20	8.0	200	200	0.005	±0.071	0.0	0.5
MMC, 10	−S9	20	6.6	200	100	0.440	±0.903	0.0	24.0[c]

Abbreviations: MMC, mitomycin C; CP, cyclophosphamide.
[a] Cells from all treatment conditions were harvested 20 hours after the initiation of the treatments.
[b] Severely damaged cells were counted as 10 aberrations.
[c] $P \leq .01$, using the Fisher exact test.

Discussion

The therapeutic potential of DMD-applicable PMO SSOs has been demonstrated in vivo, in both the dystrophic *mdx* mouse model[33] and a canine model, CXMD beagle.[34] Importantly, in a head-to-head comparison between 2′OMe and PMO in the mdx mouse, PMOs were shown to be substantially more potent than the corresponding 2′OMe phosphorothioate compound following systemic administration at similar doses.[35]

AVI-4658 is a PMO developed to restore dystrophin in certain subsets of DMD patients. In cynomolgus monkeys,

Table 5. Micronucleus Test

Treatment (20 mL/kg)	Sex	Time, h	Number of Animals	PCE/Total Erythrocytes, Mean ± SD	Change From Control, %	Number of MPCE/1000 PCE, Mean ± SD	Number of MPCE/PCE Scored
Phosphate-buffered saline	M	24	5	0.405 ± 0.14	—	0.1 ± 0.22	1/10 000
	F	24	5	0.514 ± 0.07	—	0.2 ± 0.27	2/10 000
500 mg/kg[a]	M	24	5	0.378 ± 0.15	−7	0.2 ± 0.27	2/10 000
	F	24	5	0.446 ± 0.13	−13	0.0 ± 0.00	0/10 000
1000 mg/kg[b]	M	24	5	0.405 ± 0.11	0	0.2 ± 0.27	2/10 000
	F	24	5	0.544 ± 0.05	6	0.2 ± 0.27	2/10 000
2000 mg/kg	M	24	5	0.469 ± 0.05	16	0.0 ± 0.00	0/10000
	F	24	5	0.519 ± 0.05	1	0.2 ± 0.27	2/10 000
Cyclophosphamide 50 mg/kg	M	24	5	0.395 ± 0.03	−2	11.9 ± 2.56[c]	119/10 000
	F	24	5	0.389 ± 0.08	−24	12.1 ± 6.19[c]	121/10 000
Phosphate-buffered saline	M	48	5	0.527 ± 0.05	—	0.0 ± 0.00	0/10 000
	F	48	5	0.600 ± 0.02	—	0.3 ± 0.27	3/10 000
AVI-4658 2000 mg/kg	M	48	5	0.561 ± 0.06	6	0.1 ± 0.22	1/10 000
	F	48	5	0.538 ± 0.06	−10	0.0 ± 0.00	0/10 000

[a] Dosing volume = 5 mL/kg, single intravenous injection.
[b] Dosing volume = 10 mL/kg, single intravenous injection.
[c] Statistically significant, $P \leq .05$ (Kastenbaum–Bowman tables).

following subcutaneous or intravenous administration, no test article–related effects were seen on arterial blood pressure, heart rate, ECG, respiratory parameters, global neurological activity, or renal and liver functions at the maximum feasible dose (320 mg/kg). No mutagenicity was observed in the bacterial reverse mutation assay, a CHO chromosome aberration assay, or a mouse bone marrow micronucleus assay.

Based on these data, a proof-of-concept clinical trial to evaluate the safety and efficacy of AVI-4658, delivered by intramuscular injection in DMD boys, was recently performed in the United Kingdom.[36] In this study, DMD patients, subdivided into 2 groups, received 0.09 mg (2 boys) or 0.9 mg (5 boys) of AVI-4658 in 900 μL of normal saline in one of their extensor digitorum brevis (EDB) muscles and 900 μL normal saline in the contralateral EDB. Both EDB muscles were biopsied either at 3 or 4 weeks following the injections. All safety evaluations showed no adverse events related to administration of AVI-4568. Furthermore, specific dose-dependent exon skipping was clearly demonstrated in the treated EDB muscles compared with the contralateral saline-injected muscles. Strong dystrophin production was clearly evident in the treated muscle in all individuals in the high-dose cohort. Dystrophin sarcolemmal localization suggests appropriate interaction with other members of the DGC complex.

AVI has now initiated a dose-ranging clinical study in the United Kingdom, in ambulant DMD patients in whom the safety and efficacy of repeated doses of AVI-4658, delivered intravenously, is being assessed. Enrollment is planned for up to 24 subjects in 6 cohorts. Each subject will be treated once weekly for 12 weeks and will undergo a muscle biopsy 2 weeks after the last administration. Subjects will receive AVI-4658 at 0.5, 1.0, 2.0, 4.0, 10.0, or 20.0 mg/kg per injection. Overall, this study will explore a wide (40-fold) dose range, up to relatively high doses, to thoroughly explore and understand the nature of the dose response, clinically, by assessment of expression of dystrophin and improvement of the condition in patients. The animal safety studies reported here were crucial in enabling such a study and in demonstrating the safety of AVI-4658 and the PMO class of compounds in general. Once a safe and effective dose has been established in the clinic, further confirmatory clinical studies are planned in both Europe and the United States.

Clinicians have recently tested a single 0.9-mg intramuscular dose of 2′OMe-based SSO (with a negatively charged backbone) targeted to exon 51 in 4 DMD patients.[37] As with the AVI-4658 intramuscular study, analysis of the biopsies from drug-injected tibialis anterior muscle showed evidence of accurate skipping of exon 51 and evidence of dystrophin protein expression. These same researchers are now performing an additional clinical study to investigate 2′OMe oligonucleotides following subcutaneous injections of up to 6 mg/kg for 5 weeks and recently reported dystrophin expression, although this was not quantified, with limited clinical benefit.[38] The reasons for not escalating the doses further in this study were not clear but may be related to concerns over the dose-limiting toxicities seen in preclinical studies with phosphorothioate compounds (reviewed in by Rayburn et al[13]). Further preclinical results with the 2′OMe oligonucleotides are needed before determining whether the dose-limiting toxicities of phosphorothioates will inhibit their utility in the treatment of DMD or other exon-skipping amenable diseases, especially considering the lack of preclinical toxicity seen with the AVI-4658 PMO. To

help inform decisions as to which of the 2 chemistries may better serve DMD boys and be appropriate for lifelong administration, the results contained in this article may be especially important if clinical studies for both chemistries are planned to run simultaneously.

The favorable safety profile of the uncharged PMOs has been remarkably consistent and predictable, as more than 460 patients have been safely dosed with PMO in clinical trials, for a variety of indications. This body of knowledge suggests that AVI-4658 will have the clinical safety profile characteristic of other PMO class drugs and is in direct contrast to phosphoroathioate compounds, which show class effect dose-limiting toxicities that can include mortality in preclinical testing. Importantly, the safety of PMO drugs will allow for a thorough exploration of the clinical dose response of AVI-4658, while clinical studies define the effective dose that may offer the first effective treatment of the lethal genetic childhood disease of DMD.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

Funding

The authors declared that they received no funding with respect to the authorship and/or publication of this article.

Acknowledgments

We would like to thank study directors O. Boucheix of MDS Pharma and V. O. Wagner, R. Gudi, and L. Krsmanovic of BioReliance for their efforts in performance of the safety pharmacology and bacterial reverse mutation assay, chromosome aberration test, and mouse micronucleus evaluation of AVI-4658, respectively. We would also like to thank R. Kole, S. Stadnicki, and P. Medeiros for their critical evaluation of this article.

References

1. Emery A, Muntoni F. Clinical features. In: Emery A, Muntoni F, eds. *Duchenne Muscular Dystrophy*. 3rd ed. Oxford, UK: Oxford University Press; 2003:26-45.
2. Emery AE. The muscular dystrophies. *Lancet*. 2002;359(9307): 687-695.
3. Drachman DB, Toyka KV, Myer E. Prednisone in Duchenne muscular dystrophy. *Lancet*. 1974;2(7894):1409-1412.
4. Adcock IM, Caramori G. Cross-talk between pro-inflammatory transcription factors and glucocorticoids. *Immunol Cell Biol*. 2001;79(4):376-384.
5. Dubrovsky AL, Angelini C, Bonifati DM, Pegoraro E, Mesa L. Steroids in muscular dystrophy: where do we stand? *Neuromuscul Disord*. 1998;8(6):380-384.
6. Merlini L, Cicognani A, Malaspina E, et al. Early prednisone treatment in Duchenne muscular dystrophy. *Muscle Nerve*. 2003;27(2):222-227.
7. Dunckley MG, Manoharan M, Villiet P, Eperon IC, Dickson G. Modification of splicing in the dystrophin gene in cultured Mdx muscle cells by antisense oligoribonucleotides. *Hum Mol Genet*. 1998;7(7):1083-1090.
8. Takeshima Y, Wada H, Yagi M, et al. Oligonucleotides against a splicing enhancer sequence led to dystrophin production in muscle cells from a Duchenne muscular dystrophy patient. *Brain Dev*. 2001;23(8):788-790.
9. Wilton SD, Lloyd F, Carville K, et al. Specific removal of the nonsense mutation from the mdx dystrophin mRNA using antisense oligonucleotides. *Neuromuscul Disord*. 1999;9(5):330-338.
10. van Deutekom JC, Bremmer-Bout M, Janson AA, et al. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. *Hum Mol Genet*. 2001; 10(15):1547-1554.
11. Aartsma-Rus A, Janson AA, Kaman WE, et al. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. *Hum Mol Genet*. 2003;12(8):907-914.
12. Mann CJ, Honeyman K, Cheng AJ, et al. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc Natl Acad Sci U S A*. 2001;98(1):42-47.
13. Rayburn ER, Zhang R. Antisense, RNAi, and gene silencing strategies for therapy: mission possible or impossible? *Drug Discov Today*. 2008;13(11-12):513-521.
14. Iversen PL, Cornish KG, Iversen LJ, Mata JE, Bylund DB. Bolus intravenous injection of phosphorothioate oligonucleotides causes hypotension by acting as alpha(1)-adrenergic receptor antagonists. *Toxicol Appl Pharmacol*. 1999;160(3):289-296.
15. Farman CA, Kornbrust DJ. Oligodeoxynucleotide studies in primates; antisense and immune stimulatory indications. *Toxicol Pathol*. 2003;31(suppl):119-122.
16. Sheehan JP, Lan HC. Phosphorothioate oligonucleotides inhibit the intrinsic tenase complex. *Blood*. 1998;92(5):1617-1625.
17. Swayze EE, Siwkowski AM, Wancewicz EV, et al. Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic Acids Res*. 2007;35(2):687-700.
18. Bauman J, Jearawiriyapaisarn N, Kole R. Therapeutic potential of splice-switching oligonucleotides. *Oligonucleotides*. 2009;19(1): 1-14.
19. Iversen PL. Phosphorodiamidate morpholino oligomers: favorable properties for sequence-specific gene inactivation. *Curr Opin Mol Ther*. 2001;3(3):235-238.
20. Sazani P, Graziewicz M, Kole R. Splice switching oligonucleotides as potential therapeutics. In: Crooke ST, ed. *Antisense Drug Technology: Principles, Strategies, and Applications*. 2nd ed. Boca Raton, FL: CRC Press; 2007:89-114.
21. Arechavala-Gomeza V, Graham IR, Popplewell LJ, et al. Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during dystrophin pre-mRNA splicing in human muscle. *Hum Gene Ther*. 2007;18(9):798-810.
22. Sprikkelman AB, Van Eykern LA, Lourens MS, Heymans HS, Van Aalderen WM. Respiratory muscle activity in the assessment of bronchial responsiveness in asthmatic children. *J Appl Physiol*. 1998;84(3):897-901.
23. van Lunteren E, Haxhiu MA, Deal EC Jr, Arnold JS, Cherniack NS. Respiratory changes in thoracic muscle length during bronchoconstriction. *J Appl Physiol*. 1987;63(1):221-228.

24. Clement MG, Albertini M, Aguggini G. Effects of PGF2 alpha on the EMG of costal and crural parts of the diaphragm of the newborn pig. *Prostaglandins Leukot Essent Fatty Acids*. 1991;43(3):167-173.
25. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutat Res*. 1975;31(6):347-364.
26. Green MH, Muriel WJ. Mutagen testing using TRP+ reversion in *Escherichia coli*. *Mutat Res*. 1976;38(1):3-32.
27. Preston RJ, Au W, Bender MA, et al. Mammalian in vivo and in vitro cytogenetic assays: a report of the U.S. EPA's gene-tox program. *Mutat Res*. 1981;87(2):143-188.
28. Greenwood SK, Hill RB, Sun JT, et al. Population doubling: a simple and more accurate estimation of cell growth suppression in the in vitro assay for chromosomal aberrations that reduces irrelevant positive results. *Environ Mol Mutagen*. 2004;43(1):36-44.
29. Evans HJ. Cytological methods for detecting chemical mutagens. In: Hollaender A, ed. *Chemical Mutagens: Principles and Methods for Their Detection*. Vol 4. New York, NY: Plenum Press; 1976.
30. Galloway SM, Aardema MJ, Ishidate M Jr, et al. Report from working group on in vitro tests for chromosomal aberrations. *Mutat Res*. 1994;312(3):241-261.
31. Mavournin KH, Blakey DH, Cimino MC, Salamone MF, Heddle JA. The in vivo micronucleus assay in mammalian bone marrow and peripheral blood: a report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res*. 1990;239(1):29-80.
32. Kastenbaum MA, Bowman KO. Tables for determining the statistical significance of mutation frequencies. *Mutat Res*. 1970;9(5):527-549.
33. Alter J, Lou F, Rabinowitz A, et al. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. *Nat Med*. 2006;12(2):175-177.
34. Yokota T, Lu QL, Partridge T, et al. Efficacy of systemic morpholino exon-skipping in Duchenne dystrophy dogs. *Ann Neurol*. 2009;65(6):667-676.
35. Heemskerk HA, de Winter CL, de Kimpe SJ, et al. In vivo comparison of 2′-O-methyl phosphorothioate and morpholino antisense oligonucleotides for Duchenne muscular dystrophy exon skipping. *J Gene Med*. 2009;11(3):257-266.
36. Kinali M, Arechavala-Gomeza V, Feng L, et al. Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurol*. 2009;8(10):918-928.
37. van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. *N Engl J Med*. 2007;357(26):2677-2686.
38. Goemans NM, Buyse G, Tulinius M, Verschuuren JJG, de Kimpe SJ, van Deutekom JCT. A phase I/IIa study on antisense compound PRO051 in patients with Duchenne muscular dystrophy. *Neuromuscul Disord*. 2009;19(8-9):659-660.

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EXHIBIT BN

D8
Opposition ./. EP 2 206 781 B1
Patentee: The University of Western Australia
Opponent: Nippon Shinyaku Co., Ltd.
Lederer & Keller
Patentanwälte
Unsöldstraße 2
80538 München

Experimental report

Under my supervision the following experiments have been performed in the discovery research laboratories in Tsukuba, Nippon Shinyaku Co. as described in more detail below:

Materials and Methods

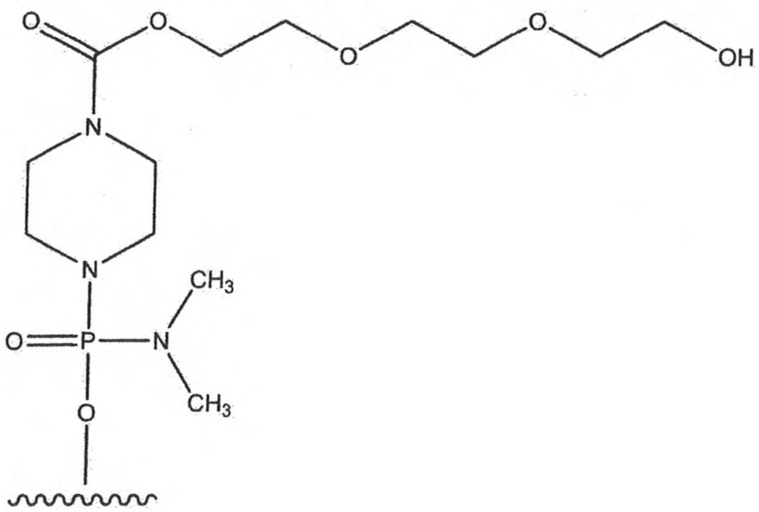
Antisense Oligomers

PMO Nos. 1–3 shown in Table 1 below, which cause skipping of the 53rd exon in the human dystrophin gene, were synthesized as described in patent no. **EP 2612917**. Each synthesized PMO was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.).

Table 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
45–62	5'-CTGTTGCCTCCGGTTCTG-3'	SEQ ID NO: 1
49–69	5'-CATTCAACTGTTGCCTCCGGT-3'	SEQ ID NO: 2
50–69	5'-CATTCAACTGTTGCCTCCGG-3'	SEQ ID NO: 3

Antisense oligomers with the above sequences, wherein the 5' end is the group of chemical formula (1) below, were prepared.



Formula (1)

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SEQ ID NO: 1	ESI-TOF-MS Calculated: 6239.18	Measured: 6238.91
SEQ ID NO: 2	ESI-TOF-MS Calculated: 7216.53	Measured: 7216.72
SEQ ID NO: 3	ESI-TOF-MS Calculated: 6886.42	Measured: 6886.83

The localization of the oligonucleotides in the relevant area is shown in the enclosed annex A

Cells

RD cells were obtained from the Health Science Research Resources Bank and cultured under 5% CO_2 at 37°C in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum.

Transfection of PMO into cells

PMOs were dissolved in distilled water and transfected into RD cells using Amaxa cell line Nucleofector kit L and a Nucleofector II device (Lonza, Basel, Switzerland) with program T-030.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from RD cells using a QIAshredder spin column (Qiagen, Valencia, CA, USA) and an RNeasy mini kit (Qiagen). RNA concentrations were determined by the absorbance at 260 nm by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed with 400 ng of the extracted total RNA using a Qiagen OneStep RT-PCR Kit (Qiagen). The forward primer was 5'-CTG AGT GGA AGG CGG TAA AC-3' and the reverse primer was 5'-GAA GTT TCA GGG CCA AGT CA-3'. RT-PCR was performed using an RTC-100 thermocycler (MJ Research, Watertown, MA, USA). The RT-PCR program was as follows: reverse transcription at 50°C for 30 min and heat denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR reaction products were analyzed using a 2100 Bioanalyzer (Agilent, Waldbronn, Germany). The skipping efficiency was determined from the expression [PCR reaction products without exon 53] × 100 / ([PCR reaction products without exon 53] + [PCR reaction products with exon 53]).

Results

The following results were measured:

Skipping efficiency

Concentration (μM)		3	10	30
SEQ ID NO: 1	45-62	0.2 ± 0.3	5.9 ± 1.3	15.1 ± 10.9
SEQ ID NO: 2	49-69	0.6 ± 0.3	2.0 ± 0.5	5.5 ± 2.0
SEQ ID NO: 3	50-69	1.4 ± 1.1	3.0 ± 3.4	7.1 ± 3.0

mean ± S.D.

This means that SEQ ID NO: 1 induced exon 53 skipping in vitro more strongly than SEQ ID NO: 2 or SEQ ID NO: 3 did. Since the activity of the various oligonucleotides differs substantially it is evident that the invention cannot be worked successfully over the whole scope of the claim.

I, Mr. Naoki Watanabe declare that the experiments have been performed under my supervision and that I have measured the results as presented in this experimental report.

Naoki Watanabe

Mr. Naoki Watanabe

Address 3-14-1, Sakura, Tsukuba, Ibaraki, Japan

Date August 15. 2016

Annex A

			23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
mRNA		5'-	G	G	A	U	G	A	A	G	U	A	C	A	A	G	A	A	C	A	C	C	U	U	C	A	G	A	A	C	C	G	G	A	G	G	C
23-47																																					
39-69																																					
45–62	SEQ ID NO: 1																																				
49–69	SEQ ID NO: 2																																				
50–69	SEQ ID NO: 3																																				
antisense		3'-	C	C	U	A	C	U	U	C	A	U	G	U	U	C	U	U	G	U	G	G	A	A	G	U	C	U	U	G	G	C	C	U	C	C	G

Annex A

58	59	60	61	62	63	64	65	66	67	68	69	
A	A	C	A	G	U	U	G	A	A	U	G	-3'
U	U	G	U	C	A	A	C	U	U	A	C	-5'

EXHIBIT BO

D 8-1

Experimental report

Under my supervision the following experiments have been performed in the discovery research laboratories in Tsukuba, Nippon Shinyaku Co. as described in more detail below:

Materials and Methods

Antisense Oligonucleotides

PMOs of SEQ ID NOs: 1–4 shown in Table 1 below, which cause skipping of the 53rd exon in the human dystrophin gene, were synthesized as described in patent no. **EP 2612917**. Each synthesized PMO was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.).

Table 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NOs:
45–62	5'-CTGTTGCCTCCGGTTCTG-3'	SEQ ID NO: 1
49–69	5'-CATTCAACTGTTGCCTCCGGT-3'	SEQ ID NO: 2
50–69	5'-CATTCAACTGTTGCCTCCGG-3'	SEQ ID NO: 3
39-69	5'-CATTCAACTGTTGCCTCCGGTTCTGAAGGTG-3'	SEQ ID NO: 4

Antisense oligomers with the above sequences, wherein the 5′ end is the group of chemical formula (1) below, were prepared.

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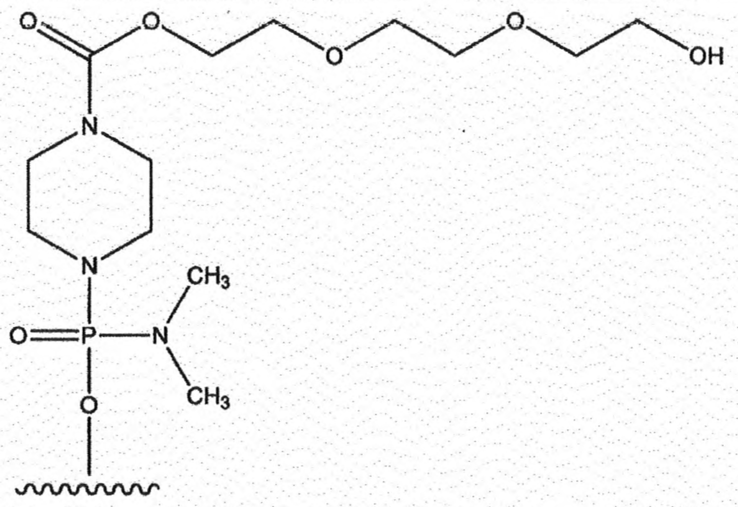

Formula (1)

SEQ ID NO: 1	ESI-TOF-MS Calculated: 6239.18	Measured: 6238.91
SEQ ID NO: 2	ESI-TOF-MS Calculated: 7216.53	Measured: 7216.72
SEQ ID NO: 3	ESI-TOF-MS Calculated: 6886.42	Measured: 6886.83
SEQ ID NO: 4	ESI-TOF-MS Calculated: 10620.69	Measured: 10620.96

The localization of the oligonucleotides in the relevant area is shown in the enclosed annex A

Cells

RD cells were obtained from the Health Science Research Resources Bank and cultured under 5% CO_2 at 37°C in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum.

Transfection of PMO into cells

PMOs were dissolved in distilled water and transfected into RD cells using Amaxa cell line Nucleofector kit L and a Nucleofector II device (Lonza, Basel, Switzerland) with program T-030.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from RD cells using a QIAshredder spin column (Qiagen, Valencia, CA, USA) and an RNeasy mini kit (Qiagen). RNA concentrations were determined by the absorbance at 260 nm by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed with 400 ng of the extracted total RNA using a Qiagen OneStep RT-PCR Kit (Qiagen). The forward primer was 5'-CTG AGT GGA AGG CGG

TAA AC-3' and the reverse primer was 5'-GAA GTT TCA GGG CCA AGT CA-3'. RT-PCR was performed using an RTC-100 thermocycler (MJ Research, Watertown, MA, USA). The RT-PCR program was as follows: reverse transcription at 50°C for 30 min and heat denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR reaction products were analyzed using a 2100 Bioanalyzer (Agilent, Waldbronn, Germany). The % skipping efficiency was determined from the expression [PCR reaction products without exon 53] × 100 / ([PCR reaction products without exon 53] + [PCR reaction products with exon 53]).

Results

The following results were measured:

% Skipping efficiency

Concentration (μM)		10	30
SEQ ID NO: 1	45-62	5.9 ± 1.3	15.1 ± 10.9
SEQ ID NO: 2	49-69	2.0 ± 0.5	5.5 ± 2.0
SEQ ID NO: 3	50-69	3.0 ± 3.4	7.1 ± 3.0
SEQ ID NO: 4	39-69	18.3 ± 3.6	24.7 ± 4.4

mean ± S.D.

This means that SEQ ID NO: 1 and SEQ ID NO:4 induced exon 53 skipping in vitro more strongly than SEQ ID NO: 2 or SEQ ID NO: 3 did. Since the activity of the various oligonucleotides differs substantially it is evident that the invention cannot be worked successfully over the whole scope of the claim.

I, Mr. Naoki Watanabe declare that the experiments have been performed under my supervision and that I have measured the results as presented in this experimental report.

Naoki Watanabe

Mr. Naoki Watanabe

Address 3-14-1, Sakura, Tsukuba, Ibaraki Japan

Date September 26, 2017

Annex A

			23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	
mRNA		5'-	G	G	A	U	G	A	A	G	U	A	C	A	A	G	A	A	C	A	C	C	U	U	C	A	G	A	A	C	C	G	G	A	G	G	C	A	A	C	A	G	U	U	G	A	A	U	G	-3'
23-47																																																		
39-69	SEQ ID NO: 4																																																	
45-62	SEQ ID NO: 1																																																	
48-69	SEQ ID NO: 5																																																	
47-68	SEQ ID NO: 6																																																	
48-68	SEQ ID NO: 7																																																	
47-67	SEQ ID NO: 8																																																	
49-68	SEQ ID NO: 9																																																	
48-67	SEQ ID NO: 10																																																	
antisense		3'-	C	C	U	A	C	U	U	C	A	U	G	U	U	C	U	U	G	U	G	G	A	A	G	U	C	U	U	G	G	C	C	U	C	C	G	U	U	G	U	C	A	A	C	U	U	A	C	-5'

Annex A

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Experimental report

The purpose of the study reported herein is to demonstrate that claimed invention in the European Patent EP2206781 includes antisense oligonucleotides (AON) that do not have sufficient skipping activity against exon 53 of the human dystrophin gene.

Under my supervision the following experiments have been performed in the discovery research laboratories in Tsukuba, Nippon Shinyaku Co. as described in more detail below:

Materials and Methods

Antisense Oligonucleotides

PMOs of SEQ ID NOs: 1, 4 and 5 to 10 shown in Table 1 below, which cause skipping of exon 53 in the human dystrophin gene, were synthesized in accordance with the method set forth in European Patent **EP 2612917**. Each synthesized PMO was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.).

Table 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NOs.
45–62	5'-CTGTTGCCTCCGGTTCTG-3'	SEQ ID NO: 1
39-69	5'-CATTCAACTGTTGCCTCCGGTTCTGAAGGTG-3'	SEQ ID NO: 4
48-69	5'-CATTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 5
47-68	5'-ATTCAACTGTTGCCTCCGGTTC-3'	SEQ ID NO: 6
48-68	5'-ATTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 7
47-67	5'-TTCAACTGTTGCCTCCGGTTC-3'	SEQ ID NO: 8
49-68	5'-ATTCAACTGTTGCCTCCGGT-3'	SEQ ID NO: 9
48-67	5'-TTCAACTGTTGCCTCCGGTT-3'	SEQ ID NO: 10

Antisense oligomers with the above sequences, wherein the 5' end is the group of chemical formula (1) below, were prepared.

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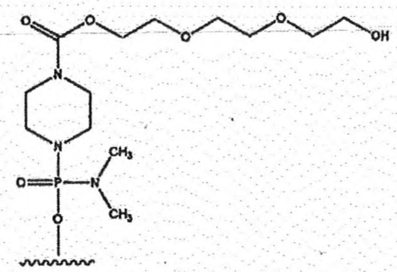

Formula (1)

SEQ ID NO: 1	ESI-TOF-MS Calculated: 6239.18	Measured: 6238.91
SEQ ID NO: 4	ESI-TOF-MS Calculated: 10620.69	Measured: 10620.96
SEQ ID NO: 5	ESI-TOF-MS Calculated: 7546.64	Measured: 7546.52
SEQ ID NO: 6	ESI-TOF-MS Calculated: 7546.64	Measured: 7546.98
SEQ ID NO: 7	ESI-TOF-MS Calculated: 7231.53	Measured: 7231.81
SEQ ID NO: 8	ESI-TOF-MS Calculated: 7207.52	Measured: 7207.04
SEQ ID NO: 9	ESI-TOF-MS Calculated: 6901.42	Measured: 6901.54
SEQ ID NO: 10	ESI-TOF-MS Calculated: 6892.41	Measured: 6892.83

The localization of the oligonucleotides in the relevant area is shown in the enclosed annex A

Cells

RD cells were obtained from the Health Science Research Resources Bank and cultured under 5% CO_2 at 37°C in Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum.

Transfection of PMO into cells

The PMOs were dissolved in distilled water and transfected into RD cells using Amaxa cell line Nucleofector kit L and a Nucleofector II device (Lonza, Basel, Switzerland) with program T-030.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from RD cells using a QIAshredder spin column (Qiagen, Valencia, CA, USA) and an RNeasy mini kit (Qiagen). RNA concentrations were determined by the absorbance at 260 nm by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RT-PCR was performed with 400 ng of the extracted total RNA using a Qiagen OneStep RT-PCR Kit (Qiagen). The forward primer was 5'-CTG AGT GGA AGG CGG TAA AC-3' and the reverse primer was 5'-GAA GTT TCA GGG CCA AGT CA-3'. RT-PCR was performed using an RTC-100 thermocycler (MJ Research, Watertown, MA, USA). The RT-PCR

program was as follows: reverse transcription at 50°C for 30 min and heat denaturation at 95°C for 15 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR reaction products were analyzed using a 2100 Bioanalyzer (Agilent, Waldbronn, Germany). The skipping efficiency was determined from the expression [PCR reaction products without exon 53] × 100 / ([PCR reaction products without exon 53] + [PCR reaction products with exon 53]).

Results

The following results were measured:

Skipping efficiency (%)

PMOs		10(μM)	30(μM)
SEQ ID NO: 1	45-62	6.0 ± 3.2	14.3 ± 4.1
SEQ ID NO: 4	39-69	15.9 ± 1.2	21.5 ± 2.7
SEQ ID NO: 5	48-69	2.8 ± 1.7	2.0 ±1.9
SEQ ID NO: 6	47-68	1.1 ± 1.9	2.5 ± 1.3
SEQ ID NO: 7	48-68	2.3 ± 2.0	2.2 ± 1.0
SEQ ID NO: 8	47-67	1.6 ± 0.8	4.6 ± 1.8
SEQ ID NO: 9	49-68	2.8 ± 0.5	2.0 ± 1.8
SEQ ID NO: 10	48-67	2.2 ± 1.4	2.6 ± 2.6

mean ± S.D.

The results clearly show that apart from SEQ ID NO: 1 and SEQ ID NO:4 having relatively high skipping activity for exon 53 in vitro, none of SEQ ID NOs: 5–10 have sufficient skipping activity. Since the activity of the tested PMOs differs substantially, it is evident that the claimed invention cannot work successfully over the whole scope of the claim.

I, Mr. Yuichiro Tone declare that the experiments have been performed under my supervision and that I have measured the results as presented in this experimental report.

Yuichiro Tone

Mr. Yuichiro Tone

Address 3-14-1, Sakura, Tsukuba, Ibaraki, Japan

Date September 26, 2017

Annex A

			23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	
mRNA		5'-	G	G	A	U	G	A	A	G	U	A	C	A	A	G	A	A	C	A	C	C	U	U	C	A	G	A	A	C	C	G	G	A	G	G	C	A	A	C	A	G	U	U	G	A	A	U	G	-3'
23-47																																																		
39-69	SEQ ID NO: 4																																																	
45–62	SEQ ID NO: 1																																																	
48-69	SEQ ID NO: 5																																																	
47-68	SEQ ID NO: 6																																																	
48-68	SEQ ID NO: 7																																																	
47-67	SEQ ID NO: 8																																																	
49-68	SEQ ID NO: 9																																																	
48-67	SEQ ID NO: 10																																																	
antisense		3'-	C	C	U	A	C	U	U	C	A	U	G	U	U	C	U	U	G	U	G	G	A	A	G	U	C	U	U	G	G	C	C	U	C	C	G	U	U	G	U	C	A	A	C	U	U	A	C	-5'

Annex A

EXHIBIT BR

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EXHIBIT BS

REDACTED

IN ITS

ENTIRETY

EXHIBIT BT

Experimental Report

(1) Methods

The following antisense oligonucleotides were synthesized in the same manner as described in the present specification (patent publication no. EP3018211) by an automated synthesizer (AKTA oligopilot plus 10 (GE Healthcare)).

H53_36-60: 5′- GTTGCCTCCGGTTCTGAAGGTGTTC -3′; corresponding to SEQ ID NO: 57 of the present application, and complementary to the 36th to the 60th nucleotides from the 5′ end of the human dystrophin gene's 53rd exon; and
H53_33-62: 5′- CTGTTGCCTCCGGTTCTGAAGGTGTTCTTG-3′; corresponding to H53A30/2 of D1, and complementary to the 33rd to the 62nd nucleotides from the 5′- end of the human dystrophin gene's 53rd exon.
H53_36-56: 5′- CCTCCGGTTCTGAAGGTGTTC -3′; corresponding to SEQ ID NO: 35 of the present application, and complementary to the 36th to the 56th nucleotides from the 5′ end of the human dystrophin gene's 53rd exon; and

The skipping efficiencies of H53_36-60 and H53_33-62 were measured in separate *in vitro* experiments but both experiments included H53_36-56 as control. Briefly, 10 μM of oligonucleotides H53_36-60, H53_33-62 and H53_36-56 were transfected with 3.5×10^5 of RD cells with an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza). The Program T-030 was used in the transfection. The transfected cells were cultured for three days in 2 mL of Eagle's minimal essential medium (EMEM)(Sigma) containing 10% fetal bovine serum (FBS) (Invitrogen) under 37°C and 5% CO_2. After washing the cells with PBS (Nissui), total RNA was extracted from RD cells using a QIAshredder spin column (Qiagen, Valencia, CA, USA) and an RNeasy mini kit (Qiagen) with a protocol suggested by the manufacturer. RT-PCR was performed with 400 ng of the extracted total RNA, using a QIAGEN OneStep RT-PCR Kit (Qiagen) and primer pairs having the nucleotide sequences of 5′-CTGAGTGGAAGGCGGTAAAC -3′ and 5′- GAAGTTTCAGGGCCAAGTCA -3′. One microliter of the RT-PCR product was analyzed with a Bioanalyzer (Agilent Technologies, Inc.).

The exon 53 skipping efficiency was calculated as below:

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$$Skipping\ efficiency(\%) = \frac{A}{(A+B)} \times 100$$

wherein "A" refers to the polynucleotide level of the band with exon 53 skipping, and "B" refers to the polynucleotide level of the band without exon 53 skipping. Experiment was repeated three times for each oligonucleotide.

(2) Results

Experiment 1

Sequences	Concentration (μM)	Repeat number	Skipping efficiency(%)	
			Mean	Standard deviation
H53_36-60	10	3	45.5	10.9
H53_36-56	10	3	68.0	1.9

Experiment 2

Sequences	Concentration (μM)	Repeat number	Skipping efficiency(%)	
			Mean	Standard deviation
H53_33-62	10	3	32.7	4.5
H53_36-56	10	3	76.2	8.0

The data show that H53_36-60 showed higher skipping efficiency in experiment 1 than H53_33-62 did in experiment 2, although control sequence of H53_36-56 showed lower skipping efficiency in test 1 than in test 2. Thus, the presently claimed oligomer H53_36-60 has superior skipping activity over H53A30/2 of D1.

D15

Declaration of Toshihiro Ueda concerning EP 3 018 211 B1

I, Toshihiro Ueda, am a Principal of Discovery Research Laboratories of Nippon Shinyaku Co., Ltd. located in Tsukuba and an expert in the field of antisense oligomers as therapeutic agents for Duchenne Muscular Dystrophy. I am the person that supervised and was responsible for the experiments in the "Experimental Report" that was filed during the examination phase of EP 3 018 211 at the European Patent Office on behalf of Nippon Shinyaku Co., Ltd. and the National Center of Neurology and Psychiatry by HGF with their letter dated 16 March 2018.

I herewith confirm that all of the antisense oligomers used in the above mentioned "Experimental Report" submitted during examination, i.e., the antisense oligomers "H53_36-60", "H53_33-62" and "H53_36-56", contained an -OH group at their 5'-end.

September 29, 2020, Tsukuba, Japan
Date, Place

Toshihiro Ueda
Name